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Detection and Molecular Characterization of Phytoplasma associated with Lethal Yellowing Disease of Coconut Palms in Cuba

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With 4 figures

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Abstract

Lethal Yellowing (LY) disease of coconut palm (*Cocos nucifera* L.) in Cuba has been reported since the end of the 19th century. In order to ascertain the presence of phytoplasmas associated with this disease, leaf samples were taken from plants showing typical disease symptoms and assayed for the LY agent by the polymerase chain reaction (PCR) using LY-specific primers. Selected PCR amplification products were cloned, sequenced and compared to that of a Mexican LY isolate from the Yucatán region. The results obtained confirm the presence of LY phytoplasma in Cuba. Cuban and Mexican isolates show an overall high degree of sequence similarity with occasional point mutations and small deletions or insertions. Based on these identified genetic differences, LY isolates from the Havana and the Yucatán region cluster together and apart from isolates originating at Maisí in eastern Cuba.

Introduction

The coconut palm (*Cocos nucifera* L.) is an important perennial oil crop of the tropics. In Cuba it is cultivated all over the country with the largest traditional areas in Baracoa (Guantánamo province) and in the municipalities of Niquero and Pilón (Granma province). Coconut cultivation is affected by various diseases that are caused by viroids, virus, fungi and bacteria (Persley, 1992). Among these, lethal yellowing (LY) in the Caribbean region represents the most important disease with respect to spread and economic loss (for more information on this disease see Center for Information on Coconut Lethal Yellowing, 1999–2001; Eden-Green and Ofori, 1997). Disease symptoms on coconut show many similarities independent of ecotype. The first symptoms are premature nutfall followed by the blackening of emerging inflorescences. This is followed by discoloration (yellowing) and necrosis of fronds that usually starts with the oldest (lowermost) leaves and progresses to the younger ones until finally the palm dies within 3–6 months after the onset of symptoms (Jones, 1997). In Cuba, LY has been recognised for more than 100 years. Coconut plants of the principal areas of cultivation were seriously affected by the disease from the end of the 19th century until the 1920's and 1930's. An epidemic outbreak of LY in the 1960's in Niquero killed the majority of plants in the area. Between 1985 and 1990 the disease destroyed the coconut cultivation (which consisted mainly of the 'criollo' coconut type) in Pilón and replanting of this area was done in 1987 and 1988.

Phytoplasmas (previously known as mycoplasma-like organisms) were identified as the probable etiological agent of LY in Jamaica during 1972 when these obligate, pleomorphic prokaryotes were consistently observed in the phloem of diseased but not healthy coconut palms by transmission electron microscopy (TEM) (Beakbane et al., 1972; Heinze et al., 1972; Plavsic-Banjac et al., 1972). In 1978, phytoplasmas were also detected by TEM in coconut affected by LY in Cuba (Waters et al., 1978). Phylogenetic analysis of 16S rDNA sequences has determined that phytoplasmas constitute a monophyletic clade of organisms within the class *Mollicutes* most closely related to the genus *Acholeplasma* (Gundersen et al., 1994). Within the clade, as many as 20 subclades (primary groups) of phytoplasmas have been delineated by these analyses (Seemüller et al., 1998). A phylogenetically based taxonomy of the phytoplasmas has been proposed in which primary groups are considered to represent distinct species for which names under the prefix 'Candidatus' are currently being assigned. Groups

identified by this method are also supported by classifications based upon restriction fragment length polymorphism (RFLP) analysis of 16S rDNA sequences amplified from phytoplasmas by polymerase chain reaction (PCR) assays (Lee et al., 1998).

Coconut lethal yellowing represents one of the primary groups resolved by phylogenetic analyses (Gundersen et al., 1994; Seemüller et al., 1998) to which several related strains have since been added (Cordova et al., 2000; Tymon et al., 1998). Transmission of the LY phytoplasma occurs via phloem-feeding insect vectors, primarily leafhoppers, planthoppers and psyllids (Wilson, 1997). The planthopper *Myndus crudus* has been identified as a primary vector of the LY phytoplasma in Florida (Howard, 1997).

The development of molecular techniques, in particular PCR assays, has allowed detection and characterization of phytoplasmas including LY agent in palms before the onset of symptom development. Molecular hybridization with specific probes has enabled detection of phytoplasmas in numerous plant hosts (Bertaccini et al., 1992; Harrison et al., 1992; Chen et al., 1992; Mpunami et al., 1997). Phytoplasma detection in plant hosts such as coconut has been enhanced by PCR amplification employing rRNA (Rohde et al., 1993; Mpunami et al., 1997; Tymon et al., 1998; Cordova et al., 2000; Eden-Green and Ofori, 1997) or nonribosomal primers (Harrison et al., 1994). This report presents the first molecular evidence by PCR with LY-specific, nonribosomal primers for the association of phytoplasma with LY disease of coconut palms in Cuba.

Materials and Methods

Sources of healthy and diseased plants

Young petioles were harvested from coconut palms showing typical LY symptoms. Palms samples were obtained from different areas in Havana City and Guantánamo province: CF-1 (Campo Florido, Havana City); BAC-1 (Bacuranao, Havana City); CF- III (Campo Florido, Havana City); VP-II (Villa Panamericana, Havana City); BAC-III (Bacuranao, Havana City); RCH–I (Chibás neighborhood, Havana City); and MAI-I (Maisí, Guantánamo). A symptomless (presumably healthy) palm MP-I (Havana City) served as a negative control.

An LY-infected coconut palm at San Miguel (Yucatán, Mexico) and four palms from Chambezi Research Station (Tanzania) showing the typical symptoms of Lethal Disease (LD) and testing positive for LD phytoplasma with rDNA primers (Rohde et al., 1993) were chosen as non-Cuban sources of phytoplasma DNA.

Extraction of DNA

Leaf samples were taken from petioles and kept for 1–2 days at an ambient temperature until DNA extraction. For each sample, 10 g of leaf material were frozen in liquid nitrogen and ground to a fine powder. Total nucleic acids were extracted by the Cetyl Trimethyl Ammonium Bromide (CTAB) method of Doyle and Doyle (1990) and treated with RNAse for the removal of RNA (Rohde et al., 1995). The integrity and approximate concentration of redissolved DNA was determined in a 0.7% agarose gel by comparison to standard DNA markers (1 kb ladder; Gibco-BRL, Groningen, The Netherlands).

PCR amplification of LY-specific sequences

For the amplification of LY DNA, nonribosomal primers LYC24F (5′-CATATTTTATTTCCTTTGCA-ATCTG-3′) and LYC24 R (5′-TCGTTTTGATAA-TCTTTCATTTGAC-3′; Harrison et al., 1994) were employed. The LY-specific PCR amplification product displays an apparent molecular size of 1 kb. Each PCR reaction mixture contained 2 μl of template DNA (20–50 ng), 1 μl of each primer (10 pmols each), 3 μl of 2 mM dNTP; 1.5 μl of 50 mM $MgCl_2$, 3 μl of 10x *Taq* polymerase buffer (Gibco-BRL, Groningen, The Netherlands) and 0.3 μl (0.5 units) *Taq* DNA polymerase (Gibco-BRL, Groningen, The Netherlands) and sterile water to a final volume of 30 μl. The PCR program consisted of an initial denaturation step (2 min at 94°C) followed by 40 cycles of step 1: 94°C, 30 s; step 2: 53°C, 50 s; step 3: 72°C, 80 s, and a final extension at 72°C for 10 min Aliquots of 10 μl were analyzed by electrophoresis though a 1% agarose gel using TAE (40 mM Tris-acetate, 1 mM EDTA) as running buffer. Products in gels were stained with ethidium bromide, visualized by UV transillumination and photographed.

Cloning and sequence analysis of LY-specific PCR products

The PCR products were purified from agarose gels using the ultrafree–DNA kit (Millipore, Eschborn, Germany) and cloned into the *Sma*I site of the pUC18 vector using standard procedures (Sambrook et al., 1989). For sequencing, plasmid DNA was isolated using the QIAGEN (Hilden, Germany) Plasmid Midiprep kit and sequenced by the ADIS unit at MPIZ on ABI377 sequencing machines. The sequence editing and analysis was carried out using the PILEUP program as part of the GCG Software package (Wisconsin, package version 10.0-UNIX). The phylogenetic analysis was performed using the PHYLIP program version 3.5c (Felsenstein, 1993).

Results and Discussion

PCR products of approximately 1 kb in size were amplified from DNA samples of LY-diseased palms from Cuba and from the positive control originating from Yucatán (Mexico), but not from DNA of healthy coconut palm (Fig. 1). Similar results were obtained by Harrison et al. (1994) for LY-infected palms in Florida with the primer combination LYC24F/LYC24R. This primer combination was derived from a cloned, anonymous, nonribosomal fragment of LY genomic DNA and detects LY from the Caribbean with high sensitivity and specificity (N. Harrison, personal communication). However, material from Tanzania showing the typical symptoms of Lethal Disease (LD) and testing positive with rDNA primers (Rohde et al.,

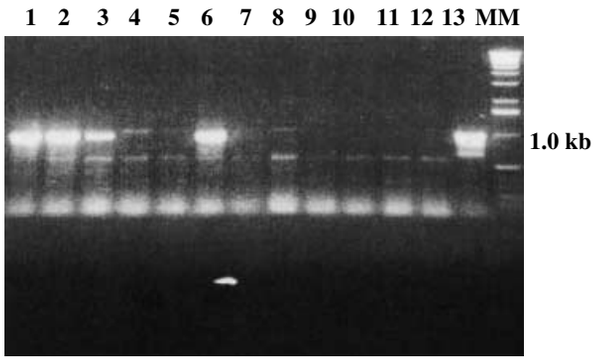

Fig. 1 PCR analysis of LY phytoplasma in young petioles of coconut plants from Cuba, Mexico and Tanzania using the primer pair LYC24F/LYC24R. Aliquots (10 μl) of PCR reactions were separated by electrophoresis in a 1% agarose gel and visualized by staining with ethidium bromide. Lane 1: CF-1; lane 2: BAC-I; lane 3: CF-III; lane 4: VP-II; lane 5: BAC-III; lane 6: RCH-I; lane 7: MP-I (negative control); lane 8 MP-II; lanes 9–12: T1, T2, T3, and T4 (Tanzania); lane 13: Mexico (positive control); lane 14: molecular weight marker (1 kb DNA ladder)

1993) did not yield the PCR amplification product (Fig. 1, lanes 9–12).

While positive results were obtained with the majority of the symptom-carrying plants from Cuba, for VP-II (Fig. 1, lane 4) and MP-II (Fig. 1, lane 8) a PCR fragment of slightly larger molecular weight and of less intensity was observed. Since it is most probable that the LY genomic region amplified by the chosen primer combination is less conserved than the rDNA region, VP-II and MP-II may harbor an LY isolate (see also sequence analysis below) to whose DNA the two primers do not bind optimally during annealing due to possible point mutations in the primer sequence region of the LY genome and which shows an insertion in the amplified region. The absence of amplification for the Tanzanian samples (Fig. 1, lanes 9–12) that tested positive for the presence of the LD agent with rDNA primers (Rohde et al., 1993) might support this interpretation. Further support for this hypothesis comes from the observations of Harrison and colleagues (N. Harrison in CICLY, 2002; Cordova et al., 2000) that newly isolated Mexican strains amplify with rDNA PCR primers, but not with nonribosomal PCR primers. Cloning and sequencing of this part of the LY genome for various isolates will help to understand the observed phenomenon. In the case of BAC-III (Fig. 1, lane 5), a plant with clear disease symptoms, the PCR analysis remained negative with respect to the expected 1 kb fragment. For this plant it was not possible to obtain young leaves due to disease progression. As has been pointed out earlier by Harrison et al. (1992), the level of

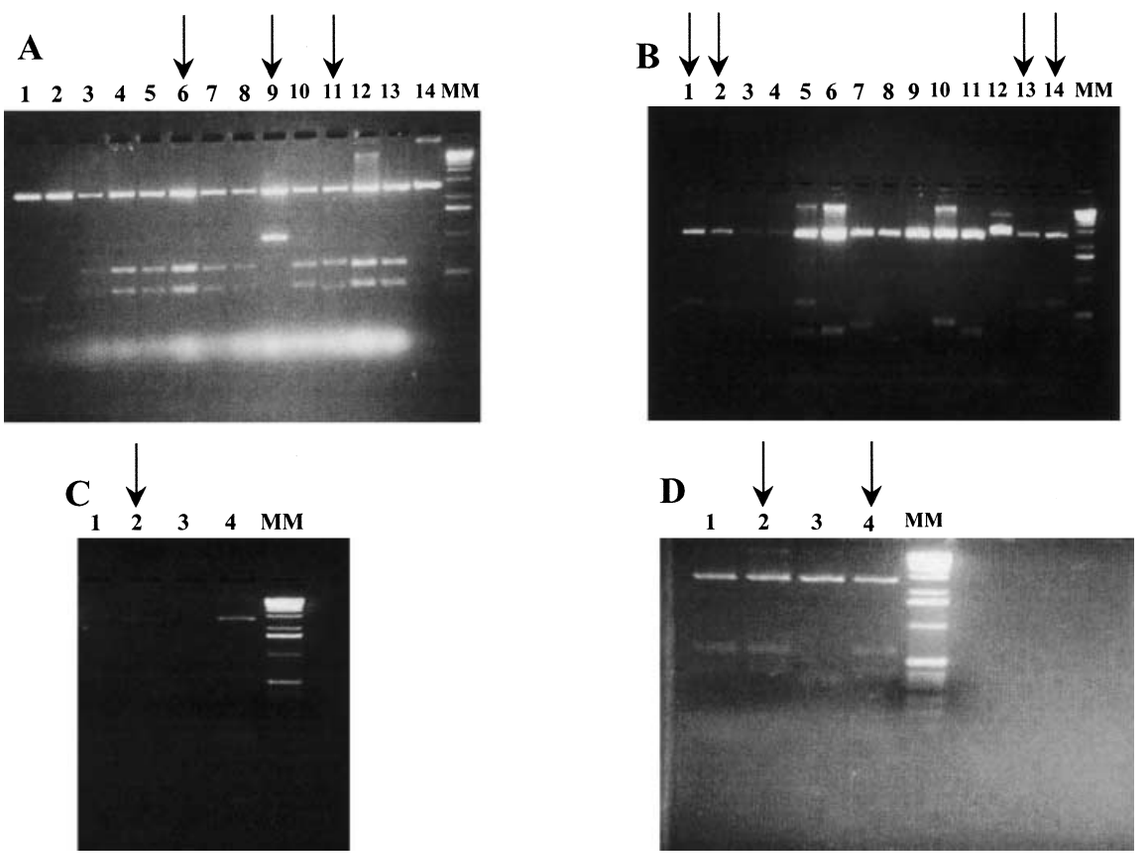

Fig. 2 Agarose gel electrophoresis of plasmid DNAs after enzymatic digestion with *Eco*RI and *Hind*III endonucleases. Plasmid DNAs were digested and the products separated by electrophoresis in a 1% agarose gel. Arrows point out the plasmids that were subjected to sequence analysis. (A) lane 6: ML15-MAI-I; lane 9: ML16-MAI-I; lane 11: ML17-MAI-I; (B) lane 1: ML34-CF-I; lane 2: ML35- CF-I; lane 13: ML43-BAC-I; lane 14: ML38-BAC-I. (C) lane 2: ML39- RCH-I. (D) lane 2: ML40-RCH-I; lane 4: ML41- RCH-I;MM (molecular weight marker; 1 kb DNA ladder)

phytoplasmas in coconut palms is considerably lower than in other susceptible species. Secondly, detection of LY phytoplasma by PCR shows highest sensitivity with the youngest leaves (Harrison et al., 1992). Furthermore, the known irregular distribution of the phytoplasmas in the phloem of infected plants and its low titers (Davis and Lee, 1992; Seemüller et al., 1998; Jones et al., 1999) increase the dependence of detection on the appropriately chosen material. We should not forget, however, that BAC-III harbors a phytoplasma distinct from LY phytoplasma.

Apart from the fact that for one of the samples (BAC-III; Fig. 1, lane 5) a positive reaction was not obtained, these first results on the PCR-based LY diagnosis in Cuba indicate a strong association of LY-like symptoms observed in the field with the presence of LY-specific PCR amplification products. The detection of phytoplasmas by PCR amplification of different regions of the LY genome such as the 16S rDNA will add to the rapid, sensitive and highly specific diagnosis of these pathogens. A first survey by general rDNA primer-based PCR further supports the presence of phytoplasma in diseased palms (unpublished).

For sequencing, PCR products obtained for LY-infected coconut palms were cloned and a total of 10 clones, ML15, ML16 and ML17 from material from Maisí; ML34 and ML35 from CF-I; ML43 and ML38 from BAC-I and ML39, ML40 and ML41 from RCH-I, were selected for sequence analysis. These clones were selected on the basis of differences in size and restriction patterns obtained after double digestion of plasmid DNAs with the enzymes *Eco*RI and *Hind*III (Fig. 2; arrows). All cloned fragments (except for ML16) contained an internal *Eco*RI restriction site (data not shown).

Figure 3 shows a sequence comparison for a segment of the approximately 1 kb PCR product for 7 Cuban clones and one from Yucatán (Mexico). Identical sequences for individual clones are not represented. Although the sequence homology among the clones studied is higher than 99%, differences are obvious also. These relate to single point mutations, but also comprise larger differences such as in the clones ML15 and ML16 isolated from the same plant in Maisí which differ by a TAATG insertion from the other clones analyzed. Remarkably, the ML16 clone contains a point mutation that abolishes the internal *Eco*RI site and the cloned insert is not cut by *Eco*RI restriction (Fig. 2A, lane 9).

Figure 4 depicts an unrooted tree for the phylogenetic relationships between the various sequenced LY isolates. Although an unequivocal interpretation of the results on sequencing may be premature at this stage, it is evident that LY isolates from the Havana region cluster together with the Yucatán isolate, excluding the Maisí isolates ML15 and ML16 obtained from the eastern tip of the Cuban island. In this context it is worthwhile to point out a possible common origin of the LY isolate from Yucatán and the City of Havana by the geographical proximity and by historical relations between Yucatán and the City of Havana. Maisí at the extreme eastern point of Cuba is some 1200 km distant from Havana and close to the Baracoa region that is the traditional region of coconut cultivation and where some 80 years ago the largest outbreak of LY occurred. Maisí is also located close to Haiti, Jamaica and other Caribbean islands where the LY disease has

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651
ML15cuba   AATATAAATA ATAATATTAA TATTAAAATA GAATAAAATT TATAAATTTT
ML16cuba   AATATAAATA ATAATATTAA TATTAAAATA GAATAAAATT TATAAATTTT
ML1mexico  AATATAAATA ATAATATTAA TATTAAAATA GAATAAAATT TATAAATTTT
ML40cuba   AATATAAATA ATAATATTAA TATTAAAATA GAATAAAATT TATAAATTTT
ML39cuba   AATATAAATA ATAATATTAA TATTAAAATA GAATAAAATT TATAAATTTT
ML41cuba   AATATAAATA ATAATATTAA TATTAAAATA GAATAAAATT TATAAATTTT
ML35cuba   AATATAAATA ATAATATTAA TATTAAAATA GAATAAAATT TATAAATTTT
ML38cuba   AATATAAATA ATAATATTAA TATTAAAATA GAATAAAATT TATAAATTTT

701
ML15cuba   TAAAAGGATC .AAAAAAAAT AATGAAAAAT AATGAAAAAT AATGATTAAA
ML16cuba   TAAAAGGATC AAAAAAAAAT AATGAAAAAT AATGAAAAAT AATGATTAAA
ML1mexico  TAAAAGGATC .AAAAAAAA. ....AAAAAT AATGAAAAAT AATGATTAAA
ML40cuba   TAAAAGGATC .AAAAAAAA. ....AAAAAT AATGAAAAAT AATGATTAAA
ML39cuba   TAAAAGGATC .AAAAAAAA. ....AAAAAT AATGAAAAAT AATGATTAAA
ML41cuba   TAAAAGGATC .AAAAAAAA. ....AAAAAT AATGAAAAAT AATGATTAAA
ML35cuba   TAAAAGGATC .AAAAAAAA. ....AAAAAT AATGAAAAAT AATGATTAAA
ML38cuba   TAAAAGGATC ..AAAAAAA. ....AAAAAT AATGAAAAAT AATGATTAAA

751
ML15cuba   ATAGAAAATG GC.AAAAATT TGATATTAGA TAACGATAGT GATTTTAATA
ML16cuba   ATAGAAAATG GC.AAAAATT TGATATTAGA TAACGATAGT GATTTTAATA
ML1mexico ATAGAAAATG GC.AAAAATT TGATATTAGA TAACGATAGT GATTTTAATA
ML40cuba   ATAGAAAATG GC.AAAAATT TGATATTAGA TAACGATAGT GATTTTAATA
ML39cuba   ATAGAAAATG GC.AAAAATT TGATATTAGA TAACGATAGT GATTTTAATA
ML41cuba   ATAGAAAATG GC.AAAAATT TGATATTAGA TAACGATAGT GATTTTAATA
ML35cuba   ATAGAAAATG GCAAAAAATT TGATATTAGA TAACGATAGT GATTTTAATA
ML38cuba   ATAGAAAATG GC.AAAAATT TGATATTAGA TAACGATAGT GATTTTAATA
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Fig. 3 Comparative analysis of LY phytoplasma DNA sequences. Sequences were aligned using the Pileup program of the GCG software package. ML15 (Maisí-Guantánamo), ML16 (Maisí-Guantánamo), ML1 (Mexico), ML40 (R. Chibás-Havana City), ML39 (R. Chibás-Havana City), ML41 (R. Chibás-Havana City), ML35 (C. Florido-Havana City), ML38 (Bacuranao-Havana City)

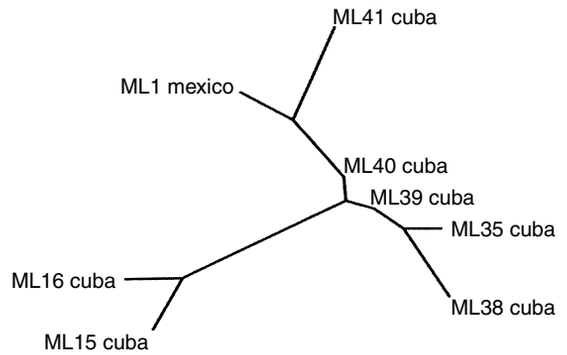

Fig. 4 Phylogenetic relationship of LY phytoplasma isolates from Cuba and Mexico. The unrooted tree was constructed for the sequenced clones from the entire sequences of 1 kb using the PHYLIP program

been known for many years. Thus the LY isolate from Maisí may represent an LY strain different from the one in Yucatán and the Havana region.

The principal goal of this work was to study for the first time by molecular analyses the presence of phytoplasma associated with LY disease of coconut palms in Cuba. PCR diagnostics for the detection of Cuban LY phytoplasmas and supported by sequence analysis confirmed the presence of this pathogen in Cuba. Furthermore, we have provided evidence for the close relationship of Cuban LY isolates from the region of Havana City to that of Yucatán that was used as a reference in our studies. Although the number of sequences determined up to now for Cuban LY isolates is low, the first results demonstrate the genetic diversity within the LY phytoplasma population resident in Cuba.

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