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16S rRNA interoperon sequence heterogeneity distinguishes strain populations of palm lethal yellowing phytoplasma in the Caribbean region

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Summary

DNA of phytoplasmas in lethal yellowing (LY)-diseased palms was detected by a nested polymerase chain reaction (PCR) assay employing rRNA primer pair P1/P7 followed by primer pair LY16Sf/LY16-23Sr. Polymorphisms revealed by *Hin*fI endonuclease digestion of rDNA products differentiated coconut-infecting phytoplasmas in Jamaica from those detected in palms in Florida, Honduras and Mexico. A three fragment profile was generated for rDNA from phytoplasmas infecting all 21 Jamaican palms whereas a five fragment profile was evident for phytoplasmas infecting the majority of Florida (20 of 21), Honduran (13 of 14) and Mexican (5 of 5) palms. The RFLP profile indicative of Florida LY phytoplasma was resolved by cloning into two patterns, one of three bands and the other of four bands, that together constituted the five fragment profile. The two patterns were attributed to presence of two sequence heterogeneous rRNA operons, rrnA and rrnB, in most phytoplasmas composing Florida, Honduran and Mexican LY strain populations. Unique three and four fragment RFLP profiles indicative of LY phytoplasmas infecting *Howea forsteriana* and coconut palm in Florida and Honduras, respectively, were also observed. By comparison, the Jamaican LY phytoplasma population uniformly contained one or possibly two identical rRNA operons. No correlation between rRNA interoperon heterogeneity and strain variation in virulence of the LY agent was evident from this study.

Key words: Phytoplasma identification, Arecaceae, coconut, 16S ribsosomal RNA, mollicute, phylogeny

Introduction

Lethal yellowing (LY) is a fast spreading, highly destructive disease of coconut and at least 35 other palm species (Harrison *et al.,* 1999). The disease has been known in parts of the western Caribbean region since the late 19th century (Eden-Green, 1997). During the last three decades, epiphytotics of LY in Jamaica and Florida have killed most of the once prevalent 'Jamaica tall' coconut palms at both localities. Spread of the disease into neighbouring regions has continued. Today, LY is most active along the Atlantic coasts of Belize and Honduras (Ashburner *et al*., 1996; Harrison & Oropeza, 1997).

Phytoplasmas are the accepted cause of LY based on their consistent detection in phloem of diseased but not healthy palms (Thomas, 1979) and based on remission of symptoms on palms in response to tetracycline therapy (McCoy *et al.,* 1983). Today, more sensitive assays using the polymerase chain reaction (PCR) have become the methods of choice for detecting obligate phytopathogenic mollicutes in diseased palms in which they usually occur in low titres (Harrison & Oropeza, 1997; Tymon *et al*., 1998). Although phytoplasmas are most reliably found in immature rather than mature tissues they may be readily detected by PCR in trunk phloem at the onset of foliar symptoms in palms (Harrison *et al*., 1999). This finding has been exploited as a means to non-destructively sample palms to confirm phytoplasma disease initially based on symptomatology (Harrison *et al.,* 2002*a*).

LY phytoplasma detection has been achieved by use of PCR assays employing primer pairs derived from rRNA sequences (Gundersen & Lee, 1996; Harrison *et al.,* 2002*a*; Lee *et al.,* 1993) or pathogen-specific non-ribosomal DNA sequences (Harrison *et al*., 1994). Additional restriction fragment length polymorphism (RFLP) or sequence analyses of rDNA amplification products also provide a means to compare, identify and classify phytoplasmas. At least 20 primary groups (putative species) (Seemüller *et al.,* 1998) and numerous subgroups of

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phytoplasmas have been delineated by these analyses (Lee *et al*., 1998). One of these is the coconut lethal yellows group, designated as group 16SrIV according to the RFLP classification scheme of Lee *et al.* (1993, 1998) or as subclade vii based on phylogenetic analysis (Gundersen *et al.,* 1994). Represented solely by a Florida strain of the LY phytoplasma in earlier classifications, other subgroup strains that include Yucatan coconut lethal decline (LDY), *Carludovica palmata* yellows (CPY) and Texas Phoenix palm decline (TPD) have since been identified and assigned to this group (Harrison *et al.,* 2002*a*).

A rehabilitation programme in Jamaica based on replacement of the local highly susceptible Jamaica tall lost to LY with resistant Malayan dwarf and hybrid MayPan (Malayan dwarf × Panama tall) resulted in an island-wide recovery of the coconut industry (Been, 1995). Both cultivars have also been widely used during replanting efforts in Florida and Mexico. Subsequently, unusually high losses of Malayan dwarfs to LY occurring at several localised sites in Jamaica and Florida were first recognised during the mid-1980s (Howard *et al.,* 1987). A re-evaluation of resistance data compiled for Malayan dwarfs and MayPans in field trials conducted in Jamaica identified significant environmental and genotype × environmental effects that influence the reaction of both cultivars to LY although factors responsible for these effects were not determined (Ashburner & Been, 1997).

Most recently, outbreaks of a disease believed to be LY have occurred around Montego Bay, Ocho Rios and Buff Bay in Jamaica where up to two-thirds of Malayan dwarfs and MayPans have been killed in these localities during the last four years. This newest development has prompted speculation about the reasons for such unexpected disease losses including the possibility that different strains of the LY agent or different vectors might be involved. The primary objective of this study was to determine the etiology of the disease currently affecting coconut palms in Jamaica. We report detection of the LY phytoplasma in all affected palms examined at this location. Based on RFLP and sequence analysis of PCR-amplified rDNA, it was also found that the LY agent in Jamaican palms varied uniformly from strains infecting palms in Florida, Honduras and Mexico.

Materials and Methods

Sources of healthy and diseased palms

A total of 21 Malayan dwarf and hybrid MayPan coconut palms displaying symptoms suggestive of LY disease were sampled at Buff Bay and Ocho Rios in Jamaica. Palms were felled and immature leaf bases adjacent to the apical meristem (heart tissues) were excised from each palm. Symptomatic palms in Yucatan, Mexico and on the grounds of the University of Florida's Fort Lauderdale Research and Education Center (FLREC) were sampled in the same manner. The latter palms included coconut ecotypes Atlantic tall, Hawaiian tall, Chowghat green dwarf, Malayan dwarf, and hybrid MayPan as well as single representatives of 16 other palm species namely, Manila palm (*Adonidia merrillii* (Becc.) Becc.), dwarf sugar palm (*Arenga engleri* Becc.), palmyra palm (*Borassus flabellifer* L.), cluster fishtail palm (*Caryota mitis* Lour.), giant fishtail palm (*Caryota rumphiana* Mart.), *Chelyocarpus chuco* (Mart.) H. E. Moore, rootspine palm (*Crysophila warsecewiczii* (H. Wendl.) H. E. Bartlett), *Cyphophoenix nucele* H. E. Moore, princess palm (*Dictyosperma album* (Bory) H. Wendl. & Drude ex Scheff.), Kentia palm (*Howea forsteriana* (C. Moore & F. Muell.) Becc.), spindle palm (*Hyophorbe verschaffeltii* H. Wendl.), Chinese fan palm (*Livistona chinensis* (Jacq.) R. Br. ex Mart.), footstool palm (*Livistona rotundifolia* (Lam.) Mart.), edible date palm (*Phoenix dactylifera* L.), cliff date palm (*Phoenix rupicola* T. Anders.) and arikury palm (*Syagrus schizophylla* (Mart.) Glassman). Fourteen symptomatic coconut palms identified on the Atlantic coast of Honduras were sampled by excising interior tissues from basal trunks as previously described (Harrison *et al*., 2002*a*). A seedling Malayan dwarf coconut palm grown in a shadehouse provided a source of healthy tissues for comparative use in the study.

DNA extractions

Immature leaf bases from palms were extracted by a phytoplasma enrichment method as previously described (Harrison *et al*., 1994). For coconut trunk tissue samples, the nucleic acid extraction procedure of Doyle & Doyle (1990) was used after first grinding 1-3 g of each sample with a mortar and pestle. Resulting nucleic-acid extracts were precipitated with ethanol, pelleted by centrifugation, dried briefly *in vacuo*, resuspended in TE (10 mM Tris, 0.1 M EDTA, pH 8) buffer containing RNase and incubated for 1 h at 37°C. DNA samples were quantified by fluorometry (TKO-100 minifluorometer, Hoefer Scientific, San Francisco, CA) and stored at 4°C before use.

Phytoplasma detection by PCR

Phytoplasma infection of palms was investigated initially by PCR employing phytoplasma-universal rRNA primers P1 (Deng & Hiruki, 1991) and P7 (Smart *et al.,* 1996). Amplifications were performed in 50 µl final reaction volumes each containing 50 ng of sample DNA template, 50 ng of each primer, 125 µM of each dNTP, 1 U of Taq DNA polymerase (Promega Corp., Madison, WI) and standard PCR buffer containing 1.5 mM $MgCl_2$ (Innis & Gelfand, 1990). P1/P7-primed PCR was performed for 30 cycles, using previously described thermal cycling parameters (Harrison *et al.,* 2001).

Products of P1/P7-primed PCR were diluted 1:40 or 1:100 with sterile deionised water and 2 µl or 4 µl of each dilution was used as template for reamplification by PCR employing nested rRNA primers LY16Sf (Harrison *et al*., 2002*a*) and LY16-23Sr (Harrison *et al*., 2002*b*). The latter assay was designed in this study to amplify a 1740-bp rDNA product consisting of the 16S rRNA gene and 16-23S rRNA spacer region from the LY phytoplasma and closely related strains. For nested PCR, the following parameters were used: denaturation for 30 s (2 min 30 s for first cycle) at 94°C, annealing for 50 s at 60°C and extension for 80 s at 72°C. Reactions were terminated after the 30 cycles with a 10 min extension step and cooled to 4°C.

Palm DNAs were also assayed by PCR (40 cycles) employing non-ribosomal primers LYF1 and LYR1 as previously described (Harrison *et al.,* 1994). This assay reliably detects the LY agent, a subgroup 16SrIV-A phytoplasma, but does not detect other 16SrIV subgroup strains (Harrison *et al.,* 2002*a*,*b*). Aliquots (5 µl or 10 µl) of each final reaction mixture were electrophoresed through 1% agarose (low EEO, Fisher Scientific) gels using TAE (40 mM Tris-acetate, 1 mM EDTA) as running buffer. Products in gels were stained with ethidium bromide (EtBr), visualised by UV transillumination and photographed.

RFLP analysis of PCR products

P1/P7-primed rDNA products amplified from an LY-diseased hybrid MayPan coconut palm (MPJ) in Jamaica, and from Atlantic tall ecotypes in Florida (ATF) and Honduras (ATH), were analysed in detail by separate digestion with restriction endonucleases *Alu*I, *Dde*I, *Dra*I, *Hae*III, *Hha*I, *Hin*fI, *Msp*I, *Rsa*I, *Sau*3AI (Promega) at 37°C, *Bst*UI (New England Biolabs, Waverly, MA) at 60°C, or *Taq*I and *Tru*9I (Promega) at 65°C, for a minimum of 16 h. Products generated from all other LY-symptomatic palms by nested PCR were digested with *Hin*fI only. Digests were electrophoresed through 8% non-denaturing polyacrylamide gels using TBE (90 mM Tris-borate, 2 mM EDTA) as running buffer. Products in gels were visualised and recorded as previously described.

Cloning and sequencing of PCR products

P1/P7 products from LY-diseased coconut palms MPJ, ATF and ATH were purified separately on spin columns (QIAquick PCR Purification Kit, Qiagen, Valencia, CA) and eluted with sterile ultrapure water. Each purified product was then cloned in vector pGEM-T (Promega) and *Escherichia coli* DH5α cells (BRL Life Technologies, Rockville, MD) according to the manufacturer's instructions. After each cloning attempt, recombinant plasmid DNA was extracted separately from 12 transformant colonies (Sambrook *et al.,* 1989) using spin columns (Wizard Plus Minipreps, DNA Purification System, Promega). Five microlitres of each 50 µl recombinant plasmid DNA preparation was diluted 1:40 with TE buffer and 2 µl of each dilution then used as template during P1/P7-primed PCR (30 cycles), as previously described. Two microlitres of each resulting PCR product was analysed by *Hin*fI digestion and electrophoresis through 8% polyacrylamide gels. Representative rDNA clones from Jamaican and Florida LY phytoplasma strains were sequenced at the University of Florida's Core DNA sequencing laboratory.

Analysis of phytoplasma rDNA sequences

Phytoplasma rDNA sequences derived from LY-diseased coconut palms MPJ and ATF were assembled and putative endonuclease recognition sites in each sequence were mapped using Vector NTI 5 Suite software (Informax Inc., Bethesda, MD). Maps were examined for concordance with restriction sites previously identified by actual enzymatic digestion. Pairwise comparisons of sequences were obtained by Gap analysis (Wisconsin package Version 10.1, Genetics Computer Group (GCG), Madison, WI). For phylogenetic analysis, 16S rDNA sequences of Jamaican and Florida LY phytoplasma strains, as well as 28 other phytoplasmas representing 14 primary phytoplasma groups according to the classification scheme of Lee *et al.* (1998) and *Acholeplasma laidlawii* (Table 1) were edited and aligned using SeqEd and PileUp programs (Wisconsin package Version 10.1). Pairwise evolutionary distances between aligned sequences were created by Distances (SeqWeb version 1.2, GCG) incorporating the Kimura 2-parameter distance correction method. A phylogenetic tree was reconstructed by Neighbor-Joining from the distance matrix using GrowTree (SeqWeb) and visualised by TreeView (Page, 1996).

Results

Phytoplasma detection by PCR

Phytoplasmas were consistently detected by P1/P7-primed PCR in tissues from 20 of 21 (95.2%) declining coconut palms sampled in Jamaica, from 13 of 14 (92.9%) Honduran coconut palms and from 19 of 21 (90.5%) Florida grown palms. Positive samples were observed as weak or moderate amplification of an rDNA product of expected size (about 1.8 kb) from DNA samples. No discernible product was amplified from DNA of seedling healthy coconut palm (data not shown). Reamplification of

P1/P7 products by PCR employing nested rRNA primer pair LY16Sf/LY16-23Sr generated an rDNA product of expected size (about 1.7 kb) from all symptomatic Jamaican (Fig. 1A) and Honduran coconut palms as well as from all Florida palms that included four new host species, namely Kentia palm (*H. forsteriana*), rootspine palm (*C. warsecewiczii*), *C. chuco* and C. *nucele* (data not shown). When phytoplasma positive DNA samples were examined by PCR incorporating primer pair LYF1/LYR1, a 1 kb product was amplified from all Jamaican (Fig. 1B), Honduran and Florida palms (data not shown) thereby confirming that palms contained the LY agent, a subgroup 16SrIV-A strain.

Phytoplasma characterisation

After P1/P7 products were separately digested with endonucleases *Alu*I, *Bst*UI, *Dde*I, *Dra*I, *Hae*III, *Hha*I, *Msp*I, *Rsa*I, *Sau*3AI, *Taq*I or *Tru*9I and compared, no differences were evident between RFLP patterns of phytoplasmas infecting coconut palms MPJ, ATF or ATH in Jamaica, Florida and Honduras, respectively (Fig. 2). However, digests with *Hin*fI produced a predominant three fragment RFLP pattern for MPJ (Fig. 2A) which differed from co-identical five fragment patterns obtained for ATF (Fig. 2B) and ATH (Fig. 2C). Unlike the pattern obtained from MPJ, the combined size estimates of *Hin*fI fragments composing patterns from ATF or

Table 1. *Description of phytoplasma 16S rDNA sequences used in this study*

Phytoplasma or associated disease	16S rRNA group-subgroup affiliation	GenBank accession no.	Reference
Michigan aster yellows (MiAY)	16SrI-B	M30790	Lim & Sears, 1989
Japanese hydrangea phyllody (JHP) (‘*Candidatus*’ Phytoplasma japonicum)	16SrI-D	AB010425	Sawayanagi *et al*., 1999
Peanut witches’-broom (PnWB)	16SrII-A	L33765	Gundersen *et al*., 1994
Witches’-broom disease of lime (WBDL) (‘*Candidatus* Phytoplasma aurantifolia’)	16SrII-B	U15442	Zreik *et al*., 1995
Papaya yellow crinkle (PpYC) ‘*Candidatus* Phytoplasma australasia’	16SrII-E	Y10097	White *et al*., 1998
Western X (WX)	16SrIII-A	L04682	Seemüller *et al.,* 1998
Clover yellow edge (CYE-C)	16SrIII-B	AF175304	Davis & Dally, 2001
Palm lethal yellowing (LY)	16SrIV-A	U18747	Tymon *et al*., 1998
Coconut lethal yellowing, Florida (LYFL-C2)	16SrIV-A	AF498309	This article
Coconut lethal yellowing, Florida (LYFL-C5)	16SrIV-A	AF498308	This article
Coconut lethal yellowing, Jamaica (LYJ-C8)	16SrIV-A	AF498307	This article
Yucatan coconut lethal decline (LDY)	16SrIV-B	Y18753	Tymon *et al*., 1998
Coconut lethal disease, Tanzania (LDT)	16SrIV-C	X80177	Tymon *et al*., 1998
Carludovica palmata yellows (CPY)	16SrIV-D	AF237615	Cordova *et al*., 2000
Texas Phoenix decline (TPD)	16SrIV-D	AF434989	Harrison et al., 2002*a*
Awka disease, Nigeria (LDN)	unclassified	Y14175	Tymon *et al*., 1998
Elm yellows (EY1)	16SrV-A	AF122910	Griffiths *et al*., 1999a
Clover proliferation (CP)	16SrVI-A	L33761	Gundersen *et al*., 1994
Ash yellows (AshY1) (‘*Candidatus* Phytoplasma fraxini’)	16SrVII-A	AF092209	Griffiths *et al*., 1999b
Loofah witches’-broom (LfWB)	16SrVIII-A	L33764	Gundersen *et al*., 1994
Pigeon pea witches’-broom (PPWB)	16SrIX-A	U18763	Tymon *et al*., 1998
Apple proliferation (AT)	16SrX-A	X68375	Seemüller *et al.*, 1998
Buckthorn witches’-broom (BWB)	16SrX-E	X76431	Seemüller *et al*., 1998
Rice yellow dwarf (RYD)	16SrXI-A	D12581	Namba *et al*., 1993
Stolbur (STOL)	16SrXII-A	X76427	Seemüller *et al.*, 1998
Australian grapevine yellows (AUGY) (‘*Candidatus* Phytoplasma australiense’)	16SrXII-B	X95706	Padovan *et al*., 1996
Mexican periwinkle virescence	16SrXIII-A	AF248960	Davis & Dally, 2001
Bermudagrass white leaf (BGWL)	16SrXIV	Y16388	Seemüller *et al.*, 1998
Hibiscus witches’-broom (HibWB) (‘*Candidatus* Phytoplasma brasiliense’)	16SrXV	AF147708	Montano *et al*., 2001
Acholeplasma laidlawii	Not applicable	M23932	Weisburg *et al*., 1989

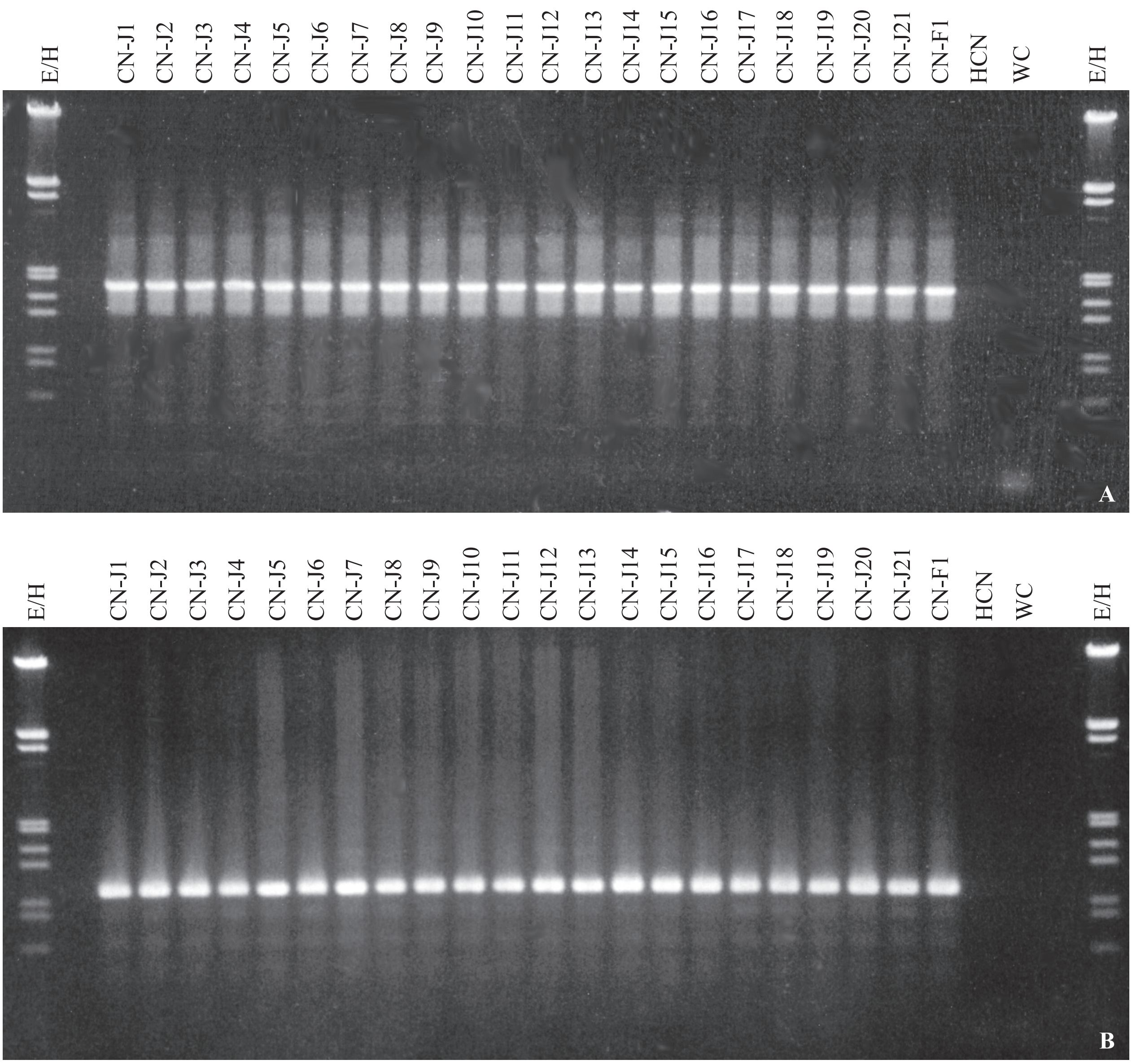

Fig. 1. Agarose gel electrophoresis of DNA products amplified by polymerase chain reaction (PCR) assays from declining coconut (*Cocos nucifera*) palms in Jamaica. (A) rDNA products (about 1.74 kb) generated by a nested PCR assay using phytoplasma universal rRNA primer pair P1/P7 followed by reamplification of products with rRNA primer pair LY16Sf/LY16-23Sr. (B) DNA products (about 1 kb) generated from declining palms by a PCR assay employing LY-specific non-ribosomal primer pair LYF1/LYR1. Template DNA samples were extracted from: CN-J = symptomatic Jamaican coconut palms; CN-F1 = LY symptomatic Florida grown coconut palm; HCN = symptomless coconut palm; WC = water control; E/H = *Hin*dIII/*Eco*RI digested λ DNA.

ATH exceeded the size of the undigested P1/P7 product (1.8 kb) from which they were derived. Thus *Hin*fI digests were repeated and extended to 40 h but resulted in no apparent changes to RFLP patterns indicating that incomplete digestion of PCR products was an unlikely cause of the rDNA profiles observed for phytoplasmas in palms ATF and ATH. Alternatively, these profiles may be explained either by dual infection of the latter palms by two dissimilar phytoplasmas or by sequence disparities between multicopy rRNA genes present in LY phytoplasma strains. Both possibilities were substantiated by further analysis of P1/P7 products cloned from LY phytoplasmas in palms MPJ, ATF and ATH.

*Hin*fI digests of rDNA products reamplified by P1/P7-primed PCR from rDNA products cloned in vector pGEM-T yielded one of two different RFLP patterns (Fig. 3). A three fragment profile was evident for products LYJ-C8, LYF-C5 and LYH-C9 cloned from palms MPJ, ATF and ATH, respectively, whereas profiles for clones LYF-C2 and LYH-C6 from palms ATF and ATH consisted of four fragments of which two were common to the first profile. Of 12 rDNA clones examined from Jamaican

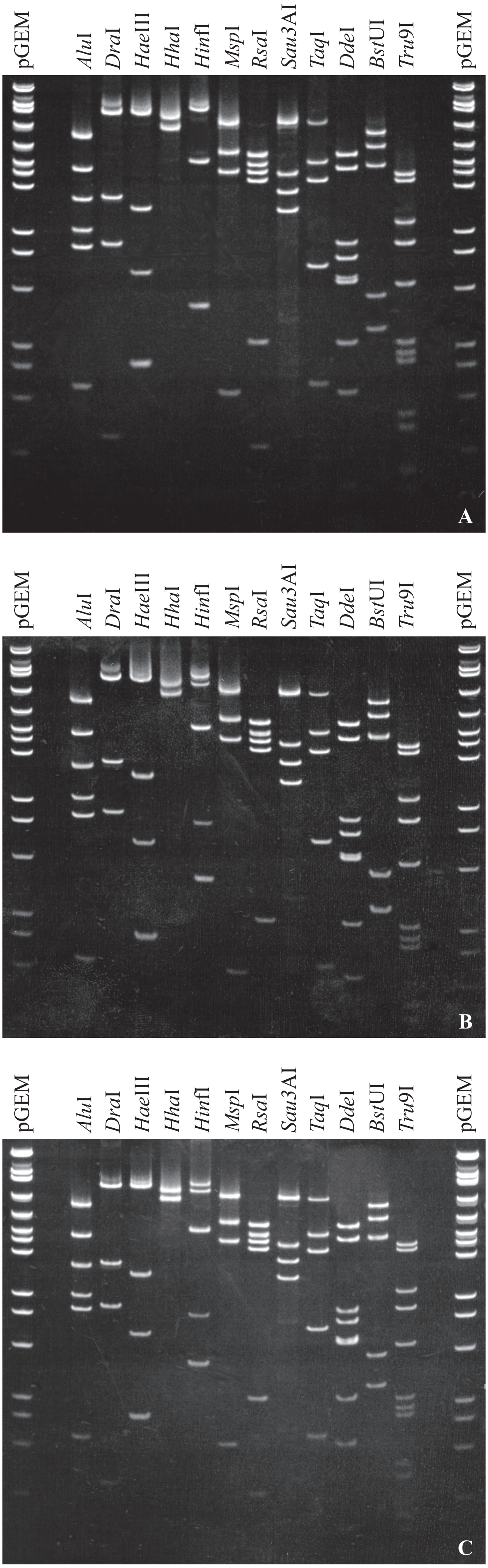

Fig. 2 (left). Restriction fragment length polymorphism patterns of rDNA products (1.8 kb) amplified from phytoplasma-infected coconut palms by a polymerase chain reaction (PCR) assay using rRNA primer pair P1/P7. Fragment patterns resulting from separate digestion of PCR products with 12 endonucleases were resolved by ethidium bromide staining after electrophoresis through an 8% polyacrylamide gel. rDNA products were amplified from DNA templates of the following lethal yellowing affected coconut palms abbreviated as follows: (A) MPJ = hybrid MayPan in Jamaica; (B) ATF = Atlantic tall in Florida; (C) ATH = Atlantic tall in Honduras. pGEM, molecular size (bp) markers in descending order: 2465, 1605, 1198, 676, 517, 460, 396, 350, 222, 179, 126, 75, 65, 51.

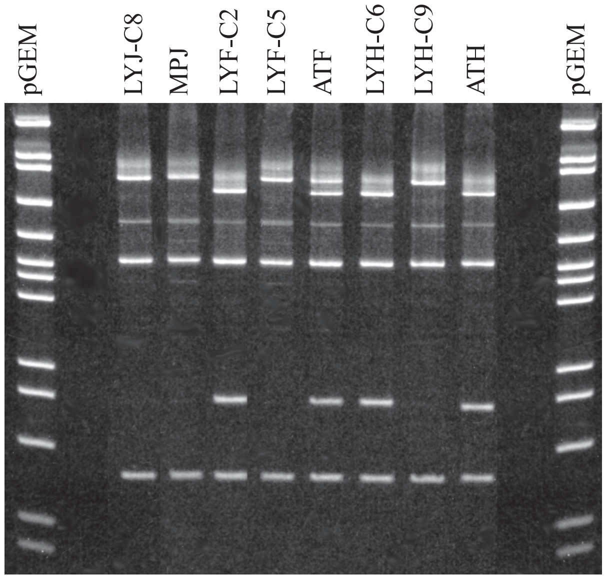

Fig. 3. *Hinf*I endonuclease restriction fragment patterns of cloned phytoplasma rDNA products (1.8 kb) amplified from lethal yellowing (LY) affected coconut palms by a polymerase chain reaction (PCR) assay employing phytoplasma universal rRNA primer pair P1/P7. LYJ-C8, LYF-C5 and LYH-C9 = three fragment RFLP profile (rrnA) generated by digestion of P1/P7 products cloned from LY-affected Jamaican (MPJ), Florida (ATF) and Honduran ATH) coconut palms, respectively; LYF-C2 and LYH-C6 = four fragment RFLP profile (rrnB) generated by digestion of P1/P7 products cloned from LY-affected Florida (ATF) and Honduran (ATH) coconut palms; MPJ, ATF, and ATH = RFLP patterns of P1/P7 products amplified directly from DNA of LY-affected Jamaican, Florida and Honduran coconut, respectively. pGEM, molecular size (bp) markers in descending order: 2465, 1605, 1198, 676, 517, 460, 396, 350, 222, 179, 126, 75, 65.

coconut MPJ, all yielded a three fragment profile as did *Hinf*I digests of P1/P7 products amplified directly from DNA of coconut palm MPJ. By comparison, P1/P7 products cloned from palms ATF and ATH yielded either three or four fragment profiles in approximately equal numbers. Collectively, the two profile types possessed equivalent size-matching fragments composing *Hin*fI digests of products amplified directly by P1/P7-primed PCR from DNAs of coconut palms ATF or ATH (Fig. 3).

*Hinf*I digests of rDNA products amplified from DNA of symptomatic Jamaican coconut palms by nested PCR employing primer pair LY16Sf/LY16-23Sr generated a three fragment RFLP profile only indicating presence of very similar or possibly the same phytoplasma strain in all of the palms analysed from this locality (Fig. 4A and B). This same analysis yielded five fragment profiles for products from phytoplasmas detected in all coconut palms in Mexico (Fig. 4B) and all but two palms sampled in Florida (Fig. 4B and C) and Honduras (Fig. 4D). Exceptions to the five fragment RFLP pattern were evident for LY phytoplasmas in a Kentia palm (HF) in Florida (Fig. 4C) for which a unique three fragment pattern was obtained and a coconut palm (CN-H5) in Honduras (Fig. 4D) which yielded a unique four fragment pattern.

The uniformity among RFLP patterns obtained for the majority of LY-diseased palms in Mexico, Florida and Honduras discounted the possibility that they were due to mixed infections by dissimilar phytoplasmas since chance infection of most palms by the same strain complex at all three separate locations seemed implausible. Rather, presence of two heterogeneous RNA operons (rrnA and rrnB) in the genome of LY phytoplasmas at these localities was judged the most likely explanation for patterns revealed by *Hin*fI digests and was supported by sequence comparisons.

Pairwise comparisons between P1/P7-primed rDNA sequences (1808 bp) LYFL-C5 (rrnA) and LYFL-C2 (rrnB) cloned from Florida LY phytoplasma strain in coconut ATF yielded a similarity value of 99.89%. Sequences rrnA and rrnB, each consisting of almost the entire 16S rRNA gene, the 16-23S rRNA spacer region and the 5'-end of the 23S rRNA gene (Schneider *et al*., 1995) varied by a total of two base substitutions in the 16S rRNA gene. In operon rrnA, these consisted of substitution of C for A at position 172 which eliminated a *Hin*fI endonuclease recognition site that was present in operon rrnB. Substitution of a T for C at position 1406 in rrnA was not detected by RFLP analysis.

Comparisons between P1/P7-primed rDNA sequence LYJ-C8 (1808 bp) from LY phytoplasma strain in Jamaican coconut MPJ and rrnA or rrnB yielded similarity values of 99.89%. Sequence LYJ-C8 differed from rrnA by a single base substitution consisting of C for T located at position 1406 and from rrnB by a single base substitution consisting of C for A at position 172 thereby eliminating a *Hin*fI restriction site that was present in rrnB. Position 172 is located within variable region 2 of the corresponding sequence of Michigan aster yellows (MiAY) phytoplasma 16S rRNA gene (Lim & Sears, 1989). Putative restriction site analysis of 16S rDNA (1524 bp) identified four *Hin*fI endonuclease recognition sites in sequence rrnB located at positions 172, 1314, 1331 and 1439, respectively, and only three sites for this endonuclease at positions 1314, 1331 and 1439, respectively, in rrnA and LYJ-C8. Putative recognition sites at positions 1314 and 1331 delineated a 17 bp fragment in sequences of all three clones which was not resolved by electrophoresis of digests on 8% polyacrylamide gels.

Phylogenetic analysis

Florida LY phytoplasma rRNA operon sequences rrnA (LYF-C5) and rrnB (LYF-C2) together with Jamaican LY phytoplasma sequence rrnA (LYJ-C8) have been deposited in the GenBank nucleotide database under accession numbers AF498308, AF498309, and AF498307, respectively. A phylogenetic distance tree was constructed from a data set which included these three sequences and comparable 16S rDNA sequences of 29 additional strains representing 15 previously established phytoplasma groups (Fig. 5). Tree branching orders resolved by the analysis were similar to and largely supported the same major phylogenetic groups identified in other recent studies (Davis & Dally, 2001; Harrison *et al*., 2002*a*). Both Florida and Jamaican coconut LY phytoplasmas clustered together with those from four previously characterised strains composing the lethal yellowing phytoplasma (16SrIV) group (Harrison *et al*., 2002*a*). Tree branching patterns verified that both phytoplasmas were evolutionarily closest to palm lethal yellowing (LY) phytoplasma and part of an existing lineage of subgroup 16SrIV-A strains.

Discussion

Recent ongoing losses of Malayan dwarf and hybrid MayPan coconut palms exhibiting symptoms of LY disease in Jamaica were attributed to the LY phytoplasma based on its consistent detection in all declining palms sampled at locations on the northern coast of the island. Although the involvement of LY was suspected based on symptoms affecting palms, the high mortality suggested some other cause since both Malayan dwarf and hybrid MayPan have exhibited satisfactory resistance to LY in Jamaica during three decades of island-wide use.

Reminiscent in intensity to the LY epiphytotic that occurred in Jamaica during the 1970s, one possible explanation for the current disease outbreak on the

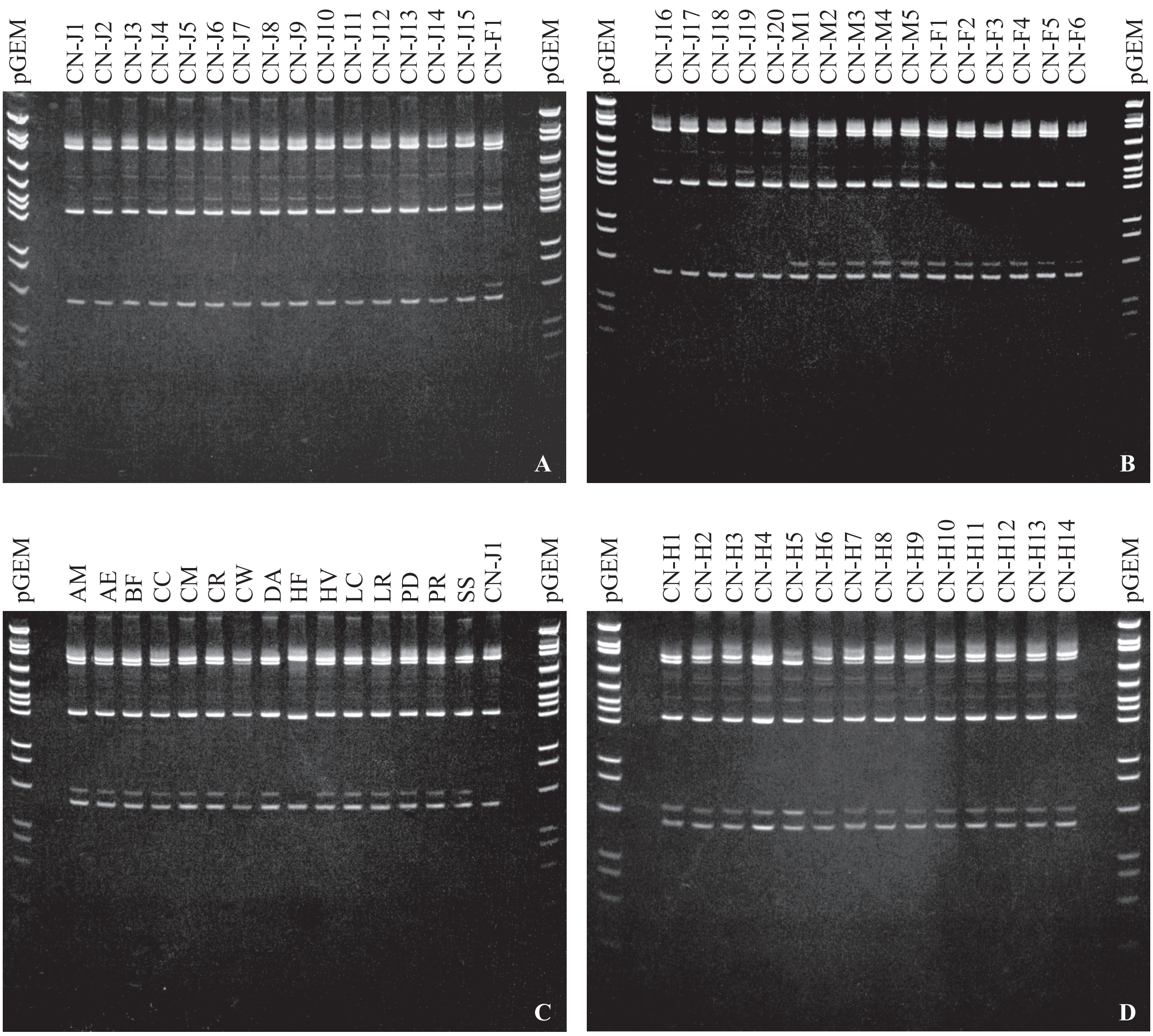

Fig. 4. Comparison of RFLP patterns resulting from *Hin*fI endonuclease digestion of rDNA products (1.7 kb) amplified from lethal yellowing (LY)-affected coconut and other palm species by a nested polymerase chain reaction (PCR) assay employing rRNA primer pairs P1/ P7 and LY16Sf/LY16-23Sr. Template DNAs for PCR were derived from the following LY-affected palms: (A) CN-J = Jamaican grown coconut palms; CN-F1 = Florida grown coconut palm; (B) CN-J = Jamaican coconut palms; CN-M = Mexican coconut palms; CN-F = Florida coconut palms; (C) AM = *Adonidia merrillii*, AE = *Arenga engleri,* BF = *Borassus flabellifer,* CM = *Caryota mitis,* CR = *Caryota rumphiana,* CC = *Chelyocarpus chuco*, CW = *Crysophila warsecewiczii*, CN = *Cyphophoenix nucele,* DA = *Dictyosperma album,* HF = *Howea forsteriana,* HV = *Hyophorbe verschaffeltii,* LC = *Livistona chinensis,* LR = *Livistona rotundifolia,* PD = *Phoenix dactylifera,* PR = *Phoenix rupicola,* SS = *Syagrus schizophylla*, CN-J = Jamaican coconut palm; (D) CN-H = Honduran grown coconut palms. pGEM, molecular size (bp) markers in descending order: 2465, 1605, 1198, 676, 517, 460, 396, 350, 222, 179, 126, 75, 65, 51.

northern coast is the involvement of a more virulent strain of the pathogen. In this work, populations of the LY phytoplasma were studied through analysis of 16S rRNA gene sequences. We used this approach as a means to assess potential strain variations within or between populations based on the hypothesis that minor differences in the highly conserved 16S rRNA gene are associated with biologically important differences in other parts of the genome of the organism. Such an assumption is supported by research on the ash yellows (AshY)-plant host pathosystem in which differing rDNA RFLP patterns of AshY phytoplasmas was correlated with variation in their aggressiveness as indicated by their ability to cause chlorosis and growth suppression of host plants (Sinclair *et al.,* 2000; Sinclair & Griffiths, 2000). In the present study, RFLP analysis of PCR-amplified rDNA sequences indicated that the LY phytoplasma population in geographically isolated Jamaica was homogeneous and varied from resident

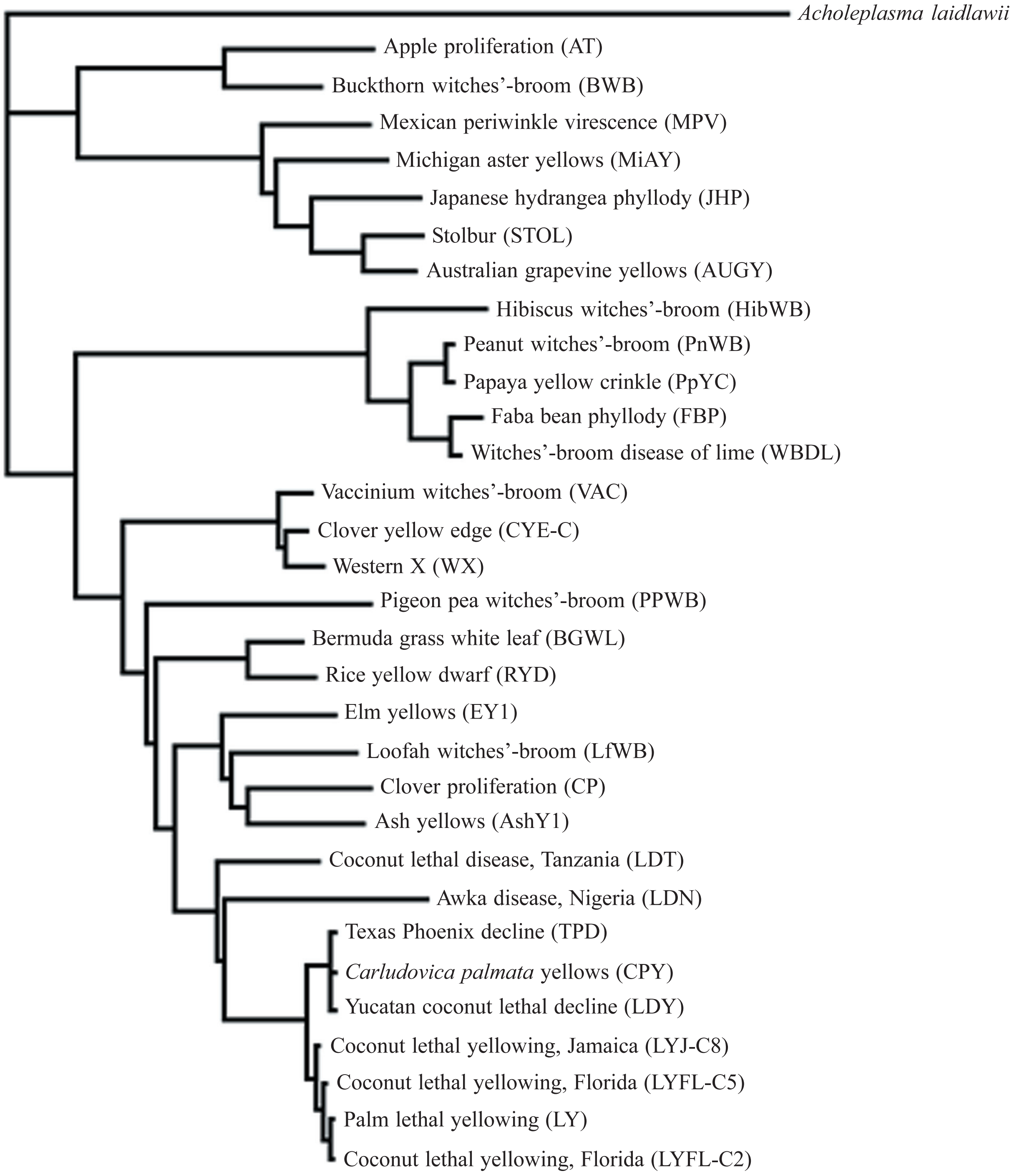

Fig. 5. Phylogenetic tree of 16S rRNA gene sequences from 30 phytoplasmas and *Acholeplasma laidlawii* constructed by the Neighbour-Joining method.

populations sampled in three disparate mainland locations in the Americas. Our comparative analysis of phytoplasma populations suggests that these differences were due to presence or absence of 16S rRNA gene heterogeneity in the LY agent. Considered a common occurrence in some bacteria (Ueda *et al.,* 1999), this trait was first recognised in clover phyllody (CPh) (Lee *et al.,* 1993) and Phormium yellow leaf (PYL) (Liefting *et al.,* 1996) phytoplasmas and may be widespread among yellows disease agents (Davis *et al.,* 1998) which are known to possess two copies of the 16S rRNA gene (Schneider & Seemüller, 1994).

The University of Florida's Fort Lauderdale Research and Education Center (FLREC) was identified as one of several localised sites in the Florida and Jamaica where unusually high losses of resistant Malayan dwarf coconut palms to LY were first recognised (Howard *et al.,* 1987). Since this earlier report, losses at the FLREC have continued and eliminated most palms of this ecotype and hybrid MayPans also from original plantings (Broschat *et al.,* 2002). Coconut palm mortality was accompanied by occasional losses of other palm species which provided sources of the LY phytoplasma for comparative use in the present study. Since Jamaican LY phytoplasma differed from Florida LY strains, this disparity indicates that 16S rRNA gene heterogeneity is probably not correlated with strain variation in aggressiveness. However, presence or absence of intra-rRNA heterogeneity may provide a useful marker for further ecological or epidemiological studies on LY. For example, the unique RFLP pattern characteristic of the pathogen in Jamaica implies that the initial appearance of the disease in neighbouring Florida, Mexico or Honduras was an unlikely consequence of spread from distant Jamaica. Conversely, the observed uniformity of RFLP patterns among the majority of strains indicates a common origin for LY phytoplasma populations on the Atlantic coast of the Americas. Furthermore, in a related study, genetic differences among LY phytoplasmas infecting coconut palms in Cuba and Yucatán, Mexico were detected following an analysis of DNA amplified by LY-specific non-ribosomal primer pair LYF1/R1, (Llauger *et al*., 2002). Extending the latter study to include comparisons of LY phytoplasma populations in Jamaica and other regions should augment our understanding of strain variability among LY phytoplasma populations.

It is also plausible that the current losses of Malayan dwarf and MayPan coconuts in Jamaica reflects changes in interactions between resident vector populations and coconut palms about which little is known. While there is convincing evidence to implicate the cixiid *Myndus crudus* Van Duzee as a primary vector of LY in Florida (Howard *et al*., 1983, 1984), similar past research failed to confirm a role for this planthopper species, or any other palm-associated species, as a vector in Jamaica (Eden-Green, 1997). Fluctuations in vector abundance, or perhaps the involvement of other vector species besides *M. crudus* might explain the increased incidence in LY disease among coconut palms at this locality and warrants further study.

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