Comparative Investigation of MLOs Associated with Caribbean and African Coconut Lethal Decline Diseases by DNA Hybridization and PCR Assays

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ABSTRACT

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Mycoplasmalike organisms (MLOs) associated with lethal decline diseases of the coconut palm (*Cocos nucifera*) in eastern and western Africa were detected by dot hybridizations using (^{32}P)dATP-labeled cloned DNA probes. Two probes, each consisting of a genomic DNA fragment of the palm lethal yellowing (LY) MLO from Florida, hybridized at moderate stringency to DNAs from four coconut cultivars with lethal disease (LD) in Tanzania and from a solitary West African Tall coconut palm with symptoms of Awka disease in Nigeria. Neither probe hybridized to DNA of the LD-affected hybrid coconut PB121 or to DNA of symptomless palms. Conserved, mollicute-specific oligonucleotide sequences used for polymerase chain reactions (PCR) primed the amplification of near full-length MLO 16S rRNA genes from all decline-affected palms. No restriction fragment length polymorphisms were observed when rDNA amplified from both LY- and LD-affected coconut palms were singly digested with the restriction endonucleases *Alu*I, *Bam*HI, *Dra*I, *Eco*RI, *Hpa*I, *Hpa*II, *Rsa*I, and *Sca*I. Polymorphisms were evident after digestion of MLO rDNA with *Taq*I. These data establish the existence of genetic relationships between MLOs associated with coconut lethal decline diseases in the western Caribbean region and in Africa and provide further evidence indicating that the LY and LD MLOs, although very similar, are not genetically identical pathogens.

Lethal yellowing (LY) is a fast-spreading mycoplasmalike organism (MLO)-associated disease of the coconut palm (*Cocos nucifera* L.) and at least 32 additional palm species (13,16,35). LY disease is endemic in several countries of the western Caribbean region (15) and is presently most active in the Yucatan peninsula of Mexico (28). Other coconut lethal declines attributed to similar etiological agents have been reported from the West African countries of Ghana (Cape St. Paul wilt), Cameroon (Kribi disease), Togo (Kaincopé), and Nigeria (Awka disease) (3,9,12). In East Africa, lethal disease (LD) is active in Tanzania (32). Overall similarities in disease syndromes have led to speculation that Caribbean LY and the African diseases may be identical (15). Conversely, important epidemiological differences that include rates of disease spread (32) and susceptibility of the LY-resistant Malayan Dwarf coconut cultivar to the African diseases (32,33) would appear to implicate the involvement of different pathogens on each continent. Until recently, however, direct comparison of coconut lethal decline MLOs has been beyond the reach of available technology.

The recent application of molecular methods to the study of MLOs has removed many of the constraints that have previously impeded progress in understanding relationships among these nonculturable plant pathogens. In particular, nucleic acid hybridizations utilizing cloned random fragments of MLO DNA as probes are providing a reliable and sensitive means for detecting MLOs in both their plant (5,13,14,18,21) and insect hosts (5,17,18,29) and for monitoring their distribution and ecology (17,19). Dot hybridizations and restriction fragment length polymorphism (RFLP) analyses of MLO chromosomal DNA have enabled differentiation of several MLOs as well as elucidation of their genetic relationships (4,20,22–24). Such comparative information has been used as a basis for delineating these pathogens into several groups or strain clusters (22,24). Amplifications of MLO genomic DNA sequences by polymerase chain reaction (PCR) assays represent the newest approach to sensitive detection of MLO DNA in plant hosts (1,8, 30). Furthermore, restriction profile analysis of PCR-amplified MLO ribosomal RNA (rRNA) gene sequences may offer a more convenient and rapid means to compare and categorize these pathogens (1,7,25,31).

In this study, the availability of cloned DNA probes for detection of the LY MLO (13) provided us with the opportunity to test the utility of these probes for detection of African coconut lethal decline agents. Further investigation of possible relationships between the LY MLO and LD MLO by PCR amplification and restriction profile analyses of their 16S rRNA genes is also reported.

MATERIALS AND METHODS

Sources of healthy and diseased palms. Tissues consisting mostly of immature leaf bases surrounding the apical meristem (heart tissues) were excised from the crowns of palms displaying early symptoms indicative of LY disease (16,35). Samples were obtained from 7- to 10-yr-old *C. nucifera* cultivars Jamaica Tall (LYJT) and Malayan Green Dwarf (LYMD), giant fishtail (*Caryota rumphiana* Mart.) palm (LYCR), silver date (*Phoenix sylvestris* (L.) Roxb.) palm (LYPS), windmill (*Trachycarpus fortunei* (Hook) H. Wendl.) palm (LYTF), and Manila (*Veitchia merrillii* (Becc.) H. E. Moore) palm (LYVM). These naturally infected palms were obtained from the palm collection located at the University of Florida's Fort Lauderdale Research and Education Center (FLREC).

Either heart or inflorescence tissue of coconut palms displaying symptoms of lethal disease (LD) (32) were obtained from the National Coconut Development Programme's Chambezi Variety Trial Grounds located in the Bagamoyo District of Tanzania during 1991. Palms were felled on site, and samples were removed from the following coconut cultivars: a Tagnanan Tall (LDTT) and an East African Tall ex Tumaini (LDEAT), both 2-yr-old seedlings; two mature Malayan Yellow Dwarfs (LDMD1 and 2); a Panama Tall (LDPT) seedling; and PB121 (LDPB), a mature IHRO hybrid (Malayan Dwarf × West African Tall), all of an undetermined age. Similar tissues were also excised from two young, asymptomatic East African Tall (HEAT) palms. Samples were initially transported to Dar es Salaam, where they were packaged in a cooler for shipment the next day to Rothamsted Experimental Station, England. Upon arrival after 3 days in transit, tissues were trimmed to remove any necroses, diced,

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and stored at −70 C.

A large unopened inflorescence was also collected from a mature West African Tall (AWAT) coconut palm located approximately 25 miles northeast of Benin City in the Ishan region of Bendel State in southeastern Nigeria. About 10% of the palm's foliage exhibited a yellow/bronze discoloration, indicating possible early-stage symptoms of Awka disease (12). Removal of the ensheathing spathe revealed blackened spikelets with discoloration extending from the tips to about 30% of spikelet length. Samples from unaffected basal tissue were prepared for shipment, as before. Tissues arrived at Rothamsted after 4 days in transit and were stored at −70 C prior to extractions.

Sources of other mollicutes. Plants affected by various other MLO-associated diseases were maintained in Florida in screenhouses. These included periwinkle (*Catharanthus roseus* (L.) G. Don) plants singly infected with periwinkle witches'-broom (FWB) (27) or pigeon pea witches'-broom (WBP) (14). Screwpine (*Pandanus utilis* DeBory) with early-stage symptoms of decline (PD) disease (36) and periwinkle with an uncharacterized virescence disease (FPV) were both collected from the grounds of the FLREC. Periwinkle with eastern aster yellows (EAY) and with western X disease (WX) were kindly provided by J. A. Wyman (University of Wisconsin, Madison) and B. C. Kirkpatrick (University of California, Davis), respectively. The Cocos spiroplasma (CS) isolate N525 (11) was kindly provided by R. E. Whitcomb (USDA-ARS, Beltsville, MD) and cultured in C3-G medium (26). *Acholeplasma oculi* (19L) and *A. axanthum* (S743), both NIAID reference strains, and an unidentified *Acholeplasma* sp. (J233), originally isolated from tissues of an LY-affected coconut palm in Jamaica (10), were kindly provided by J. G. Tully (Frederick Cancer Research Facility, Frederick, MD) and were cultured in SP-4 medium (37).

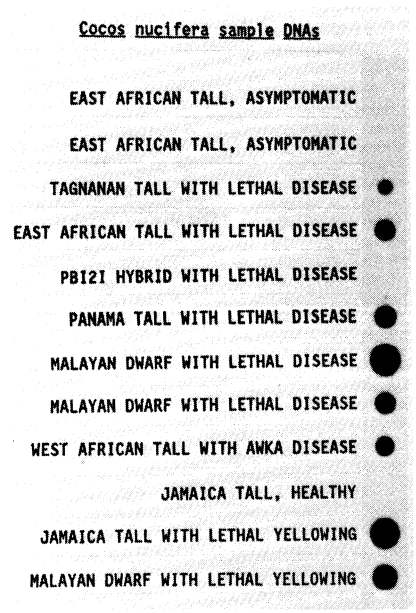

Fig. 1. Moderately stringent dot hybridization of ^{32}P-labeled probe LYI43, a 2.3-kbp *Eco*RI chromosomal DNA fragment of the lethal yellowing (LY) mycoplasmalike organism to DNAs derived from coconut palms affected by lethal disease in Tanzania, Awka disease in Nigeria, and LY disease in Florida. Each coconut sample represents 2 μg of undigested DNA.

DNA extractions. DNA extracts were prepared from tissues of healthy plants and plants with MLO-associated diseases as previously described (13). Freshly harvested tissues (50 or 100 g) were briefly ground with a Waring blender in ice-cold PS (18) extraction buffer (100 mM K_2HPO_4, 31 mM KH_2PO_4, 0.3 M sucrose, 44 mM fructose, 0.15% bovine serum albumin [fraction V], 2% polyvinylpyrrolidone [PVP-40], 30 mM ascorbic acid, 10 mM EDTA), using 4 ml of buffer per gram of tissue. The slurry was strained through cheesecloth, and the extract was clarified by centrifugation at 3,000 *g* for 10 min; then the MLO-enriched preparations were pelleted from the supernatant by centrifugation at 20,000 *g* for 30 min. Nucleic acids were extracted from pellets by the procedure of Dellaporta et al (6), then resuspended in TE buffer (10 mM Tris-HCl, pH 8.0, 1 mM EDTA) containing 50 μg ml^{-1} RNAse and incubated for 1 hr at 37 C. DNA solutions were then stored at 4 C. Cells from both spiroplasma and acholeplasma cultures were harvested by centrifugation at 20,000 *g* for 30 min at 4 C. Pellets were resuspended in 100 mM Tris (pH 8), 50 mM EDTA, 500 mM NaCl buffer containing 1% sodium dodecyl sulfate (SDS), and DNA was extracted as described above.

For African palm samples, 10 or 25 g of frozen tissue was pulverized in liquid nitrogen with a mortar and pestle. The resulting powder was resuspended in PS extraction buffer, briefly mixed to a slurry, chilled on ice for 30 min, and then strained through cheesecloth. After differential centrifugation of the filtrate, nucleic acids were extracted from MLO-enriched pellets as described previously. Nucleic acids in 75% ethanol were shipped to the FLREC, where they were pelleted by addition of a 0.1 volume of 3 M sodium acetate (pH 5.2) and centrifugation at 20,000 *g* for 15 min. Pellets were washed with 70% ethanol, briefly dried, resuspended in TE buffer (pH 8) containing RNAse, and incubated at 37 C for 1 hr.

DNA hybridizations. For dot hybridizations, DNAs were diluted in 6× SSC (1× SSC = 150 mM NaCl, 15 mM sodium citrate, pH 7.0), denatured by boiling with NaOH for 5 min, cooled on ice, and then neutralized with 2 M Tris-HCl (pH 7.0) (21). Approximately 2 μg of each DNA was blotted onto nylon membranes (Nytran; Schleicher & Schuell, Keene, NH) as a single spot by using a Bio-Dot manifold (Bio-Rad Laboratories, Richmond, CA). Membranes were air-dried, baked at 80 C for 30 min, and washed in 0.1× SSC, 0.5% SDS at 65 C for 1 hr immediately prior to prehybridization and hybridization with selected DNA probes.

Cloned probes LYD37 and LYI43 consisting of 2.2- and 2.3-kb chromosomal DNA fragments, respectively, of a Florida isolate of the lethal yellowing agent from Manila (*V. merrillii*) palm were used individually to screen dot blots under conditions of moderate stringency. These probes were chosen for hybridizations on the basis of their detection specificity. Probe LYD37 hybridizes broadly to mollicute DNAs, whereas probe LYI43 exhibits a much narrower range of specificity for detection of MLOs (13).

Membranes were prehybridized at 68 C for 16 hr in 6× SSC containing 10× Denhardt's solution (0.1% Ficoll, 0.1% polyvinylpyrrolidone, 0.1% bovine serum albumin), 0.5% SDS, and 1 mg ml^{-1} denatured salmon sperm DNA. Blots were hybridized with denatured probes at 68 C for 16 hr in the same solution and then washed at moderate stringency consisting of two washes in 2× SSC, 0.1% SDS at 25 C (30 min each wash), one wash in 0.2× SSC, 0.1% SDS at 55 C (30 min), and once again in 0.2× SSC, 0.1% SDS at 25 C (30 min). Membranes were sealed in plastic wrap and exposed to Konica PB7 blue sensitive x-ray film (Konica Medical Corp., Wayne, NJ) with intensifier screens (Lightning Plus; Du Pont, Newark, DE) for a minimum of 16 hr at −75 C.

DNA amplifications. A pair of oligonucleotide sequences (P1 and P6) used previously for PCR to prime the amplification of near full-length 16S rRNA genes of several mollicutes (7) were synthesized for our use by the DNA synthesis Core laboratory at the University of Florida's Interdisciplinary Center for Biotechnology Research. Template DNAs from lethal decline-affected or healthy palms, plants with other MLO-associated diseases, and mollicute cultures were first quantified by fluorometry (TKO-100 minifluorometer, Hoefer Scientific Instruments, San Francisco, CA), then diluted to a final concentration of 25 ng μl^{-1} with TE (pH 7.4) buffer.

Amplifications were performed in 50-μl reaction volumes, each containing 50 ng of template DNA, 50 ng of each primer, 125 μM of each dNTP, 1.5 units of AmpliTaq DNA polymerase (AS), and recommended PCR buffer (Perkin-Elmer Cetus, Norwalk, CT). Reaction mixtures were overlaid with 50 μl of mineral oil, and PCR was then performed for 30 cycles in a thermocycling oven (Bioscycler; Bios Corp., New Haven, CT) using previously described stringent (58 C annealing temperature)

thermocycling conditions (7).

Analysis of PCR amplification products. For preliminary analyses of PCR products, 10 μl of each reaction mixture was electrophoresed through 1% agarose (low EEO grade, Fisher Scientific, Pittsburgh, PA) gels using 1× TAE (40 mM Tris-acetate, 1 mM EDTA), followed by staining with ethidium bromide and visual examination upon UV transillumination. Subsequently, 10 μl-aliquots of amplification reaction mixtures were digested by addition of 1 μl of undiluted restriction enzyme and appropriate buffer followed by incubation for a minimum of 4 hr at 37 C. Twenty-one restriction enzymes—*Alu*I, *Ava*II, *Bam*HI, *Bcl*I, *Bgl*I, *Bgl*II, *Dra*I, *Eco*RI, *Eco*RV, *Hin*dIII, *Hpa*I, *Hpa*II, *Kpn*I, *Pst*I, *Rsa*I, *Sal*I, *Sca*I, *Sma*I, *Taq*I, *Xba*I, and *Xho*I (Promega Corp., Madison, WI)—were initially tested by this means, and products of digests were evaluated by electrophoresis through 1% agarose gels. A more detailed examination of DNA fragment patterns produced by selected restriction enzymes was subsequently achieved by electrophoresis of digests through 3% NuSieve GTG agarose (FMC BioProducts, Rockland, ME) gels with 1× TBE (90 mM Tris-borate, 2 mM EDTA) as running buffer.

RESULTS

DNA hybridizations. Both cloned probes LYD37 and LYI43 hybridized at moderate stringency to DNAs derived from LY-affected *C. nucifera* cvs. Jamaica Tall and Malayan Green Dwarf and to DNAs from five of six samples from coconut cultivars with symptoms of lethal disease from Tanzania. Probes also hybridized to DNA from a solitary Awka disease-affected coconut palm from Nigeria. Comparable results were obtained with each probe, and signals representative of twice-repeated hybridizations are illustrated in Figure 1. No hybridizations were observed between either probe and sample DNAs from the PB121 hybrid coconut with LD symptoms, a healthy shadehouse-grown coconut palm from Florida, and two symptomless landscape-grown Tanzanian coconut palms. Differences in signal intensities were evident between DNA samples. Hybridizations between probes and African DNAs were mostly weaker than those observed for sample DNAs derived from LY-affected palms included as positive controls. These differences were attributed to reflect either lower titers of MLO DNA in African coconut DNA samples or the degree of sequence similarity between probes and test DNAs, or both.

PCR amplification and RFLP analysis of 16S rRNA genes from coconut lethal decline MLOs and other mollicutes. A prominent 1.5-kbp DNA band was resolved by agarose gel electrophoresis from all reaction mixtures containing template DNA derived from lethal decline-affected palms. Likewise, amplified rDNA of an equivalent size was detected when PCR templates consisted of either spiroplasma or acholeplasma DNAs (Fig. 2A). No comparable product was amplified from DNAs of healthy or asymptomatic coconut palms. Weak amplification of a second, larger DNA

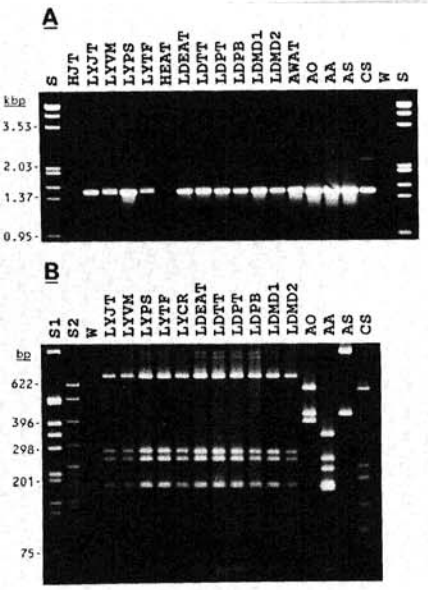

Fig. 2. Polymerase chain reaction (PCR) amplification of a 1.5-kbp 16S rDNA sequence from palms affected by various mycoplasmalike organism-associated lethal decline diseases and from culturable palm-associated mollicutes. Results from electrophoresis of **(A)** undigested PCR amplification products in a 1% low EEO agarose gel and **(B)** *Alu*I-digested PCR products in a 3% NuSieve GTG agarose gel after staining with ethidium bromide. DNA templates for PCR were derived from lethal yellowing (LY)-affected palms in Florida (LYJT = *Cocos nucifera* cv. Jamaica Tall, LYVM = *Veitchia merrillii*, LYPS = *Phoenix sylvestris*, and LYTF = *Trachycarpus fortunei*), from lethal disease (LD)-affected *C. nucifera* cultivars in Tanzania (LDEAT = East African Tall, LDTT = Tagnanan Tall, LDPT = Panama Tall, LDPB = PB121 hybrid, and LDMD1 and 2 = Malayan Yellow Dwarfs), and from an Awka disease-affected *C. nucifera* cultivar in Nigeria (AWAT = West African Tall). Additional palm template DNAs were from HJT (a shadehouse-grown healthy *C. nucifera* cv. Jamaica Tall) and HEAT (a symptomless *C. nucifera* cv. East African Tall) or from cultures of AO (*Acholeplasma oculi*), AA (*A. axanthum*), AS (*Acholeplasma* sp., isolate J233), and CS (*Cocos spiroplasma*). W = water control. DNA molecular size markers: S = *Eco*RI-*Hin*dIII digest of lambda DNA, S1 = BRL 1-kb ladder, S2 = *Msp*I-digested pBR322.

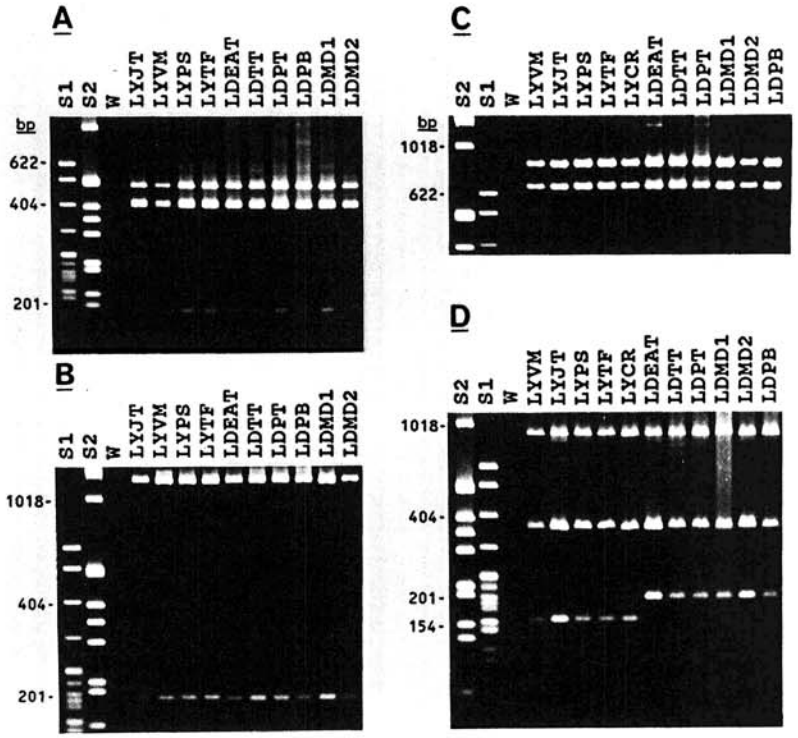

Fig. 3. Restriction profile analysis of 16S rDNA amplified by polymerase chain reaction (PCR) from landscape palms of the western Caribbean region and eastern Africa affected by mycoplasmalike organism-associated lethal decline diseases. Amplification products were digested with **(A)** *Rsa*I, **(B)** *Dra*I, **(C)** *Eco*RI, or **(D)** *Taq*I, electrophoresed in 3% NuSieve GTG agarose gels and stained with ethidium bromide. PCR template DNAs were derived from several palm species in Florida with lethal yellowing (LY) disease (LYJT = *Cocos nucifera* cv. Jamaica Tall, LYVM = *Veitchia merrillii*, LYPS = *Phoenix sylvestris*, LYTF = *Trachycarpus fortunei*, and LYCR = *Caryota rumphiana*) or from various *C. nucifera* cultivars in Tanzania with lethal disease (LD) (LDEAT = East African Tall, LDTT = Tagnanan Tall, LDPT = Panama Tall, LDPB = PB121 hybrid, and LDMD1 and 2 = Malayan Yellow Dwarfs). W = water control. DNA molecular size markers: S1 = *Msp*I-digested pBR322, S2 = BRL 1-kb ladder.

product was frequently, but not always, observed when reactions contained template DNA from either the LY-affected coconut cultivar Jamaica Tall or the *Cocos* spiroplasma.

Initial restriction profile analyses revealed no evidence for digestion of the prominent 1.5-kbp DNA product amplified from any decline-affected palms upon treatment with the restriction enzymes *Ava*II, *Bcl*I, *Bgl*I, *Bgl*II, *Eco*RV, *Hin*dIII, *Kpn*I, *Pst*I, *Sal*I, *Sma*I, *Xba*I, or *Xho*I (*data not shown*). By comparison, *Alu*I, *Bam*HI, *Dra*I, *Eco*RI, *Hpa*I, *Hpa*II, *Kpn*I, *Pst*I, *Rsa*I, *Sca*I, and *Taq*I were each found to cleave this product at least once. No differences were evident between rDNA fragment patterns of Florida and Tanzanian decline-affected palms after digestion with *Alu*I. However, this enzyme clearly distinguished rDNA of diseased palms from rDNA amplified from other culturable palm-associated mollicutes (Fig. 2B). Restriction fragment patterns resulting from *Bam*HI, *Hpa*I, *Hpa*II, *Sca*I (*data not shown*), *Rsa*I, *Dra*I, and *Eco*RI (Fig. 3A–C) digests of rDNA products from diseased Florida and Tanzanian palms were also identical. RFLPs were evident only when rDNA of palms from each geographic locality was digested with *Taq*I (Fig. 3D).

*Alu*I digests were also used to compare MLO rDNA from lethal decline-affected coconut palms with similar rDNA products amplified by PCR from other MLO-infected plants. Collectively, five different rDNA restriction fragment patterns were observed, and representative patterns are shown in Figure 4. No difference was apparent between the fragment profile obtained for the PD MLO and those of the coconut lethal decline agents. However, polymorphisms delineated the coconut decline MLOs from each of the remaining MLOs. Differences in RFLP patterns were most marked between coconut MLOs and the MLOs associated with eastern aster yellows, Florida periwinkle virescence, and periwinkle witches'-broom disease.

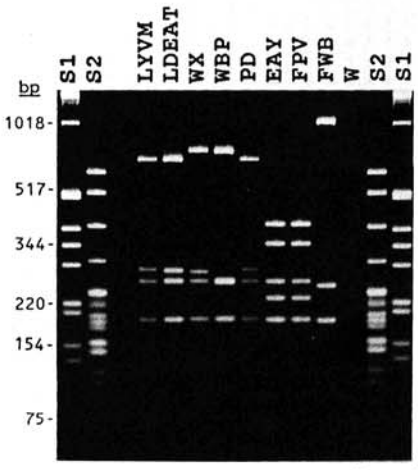

Fig. 4. *Alu*I restriction fragment profiles of 16S rDNA amplified by polymerase chain reactions (PCR) from lethal decline-affected palms and other plants with mycoplasmalike organism-associated diseases. rDNA digests were electrophoresed in 3% NuSieve GTG agarose gels and stained with ethidium bromide. PCR template DNAs were derived from LYVM = lethal yellowing-diseased *Veitchia merrillii* palm, LDEAT = lethal disease-affected *C. nucifera* cv. East African Tall, WX = western X-infected periwinkle (*Catharanthus roseus*), PD = decline-affected *Pandanus utilis*, and from periwinkle with WBP = pigeon pea witches'-broom, EAY = eastern aster yellows, FPV = Florida periwinkle virescence, and FWB = Florida periwinkle witches'-broom. W = water control. DNA molecular size markers: S1 = BRL 1-kb ladder, S2 = *Msp*I-digested pBR322.

DISCUSSION

Hybridization of LY MLO DNA probes to DNAs extracted from coconut palms affected by lethal declines in both eastern and western Africa strongly supports previous evidence that MLOs are the most likely cause of these diseases (2,9,12,32) and reveals for the first time the existence of genetic similarities between these MLOs. The involvement of genetically similar MLOs on each continent is, perhaps, not unexpected, since these pathogens collectively infect and induce superficially similar disease syndromes on the same host palm species (15). Although our results would appear to discount the involvement of genetically disparate MLOs, evidence of dissimilar MLOs inducing similar symptoms on a mutual plant host has been reported (34). Thus, the possible association of additional, as yet uncharacterized MLOs with African coconut decline diseases cannot be ruled out.

We attempted to show possible genetic relationships among the coconut lethal decline MLOs by use of genomic RFLP analysis in this study. However, preliminary results proved to be inconclusive (*unpublished data*). This was due to the availability of only small quantities of African sample DNAs and to seemingly low titers of detectable MLO DNAs within these samples. In an attempt to overcome these limitations, further comparative investigation of Caribbean and African coconut lethal decline MLOs was done using amplification of 16S rRNA genes of these organisms by PCR and subsequent restriction profile analyses of the amplified genes. However, RFLP analyses were limited to only coconut decline MLOs from Florida and Tanzania for which multiple samples were available. Use of this approach to compare MLOs and to indicate possible MLO group affiliations for taxonomic purposes has recently been demonstrated (1,25,31).

For PCR primers, we chose a pair of oligonucleotide sequences that shared considerable homology with highly conserved regions of mollicute 16S rRNA genes (7). We considered these primers to have potential for enabling amplification of 16S rDNA from many, perhaps all, MLOs. Subsequent amplifications of a DNA band of the predicted size (1.5 kbp) from template DNAs of decline-affected palms, but not from healthy palms, supported our hypothesis. Furthermore, amplification of a PCR product from DNA of the hybrid coconut PB121, a sample previously judged not to contain MLO DNA by lack of hybridization to DNA probes, clearly underscored the potential of PCR for increasing the sensitivity of detecting MLO infections of woody perennial plant hosts, such as palms, which have very low titers of the pathogen.

Both helical (11) and nonhelical (10) mollicutes have been isolated from necrotic coconut flower and heart tissues of LY-symptomatic coconut palms. The frequency of association of these culturable procaryotes and LY is uncertain, and they are likely to be excluded as surface contaminants during the extraction of DNA from diseased palm tissues by avoiding necroses (13). However, since the primers and PCR conditions used during this study permitted amplification of both spiroplasma and acholeplasma rDNA, the possibility that PCR products from diseased palms arose from such contaminant mollicute DNAs was investigated. Restriction banding patterns of amplified rDNA of known culturable palm-associated mollicutes were clearly distinct from those of rDNA that we attributed to coconut lethal decline MLOs. Thus, we found no evidence that nontarget mollicutes were amplified during our PCR experiments with palms.

Ahrens and Seemüller (1) considered *Alu*I digests of amplified MLO rDNA to provide useful molecular markers that reflected taxonomic differences among MLOs. Using this approach for the purpose of comparing the coconut lethal decline agents, we found no difference between the Florida LY MLO and the Tanzanian LD MLO. However, this enzyme distinguished the coconut decline MLOs from most others that were included for comparative purposes. rDNA fragment profiles revealed both coconut decline MLOs to closely resemble the MLO associated with *Pandanus* decline and, to a lesser extent, the MLOs implicated in western X and pigeon pea witches'-broom diseases. Differences were most apparent between coconut decline MLOs and the EAY MLO and other MLOs found naturally infecting periwinkle in southern Florida. These latter agents are all associated with diseases of herbaceous plants and collectively induce floral abnormalities rather than decline-type symptoms on their respective hosts (21,27).

Our inability to differentiate between the LY and LD MLOs with all but one of the 21 rDNA-enzyme combinations tested during this study suggests these

two pathogens are quite similar, although not identical. This conclusion is also supported by recent partial sequence analysis of the rRNA operon of both pathogens (2). However, because regions of sequence divergence in this evolutionarily conserved MLO gene between these MLOs were also identified, further differentiation of the LY MLO and the LD MLO is probable.

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