DNA Probes for Detection of Mycoplasmalike Organisms Associated with Lethal Yellowing Disease of Palms in Florida

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ABSTRACT

Harrison, N. A., Bourne, C. M., Cox, R. L., Tsai, J. H., and Richardson, P. A. 1992. DNA probes for detection of mycoplasmalike organisms associated with lethal yellowing disease of palms in Florida. Phytopathology 82:216-224.

Five *Eco*RI restriction fragments consisting of chromosomal DNA of the mycoplasmalike organism (MLO) associated with lethal yellowing (LY) disease of Manila palm (*Veitchia merrillii*) in Florida were cloned and identified. When used individually as [^{32}P]dATP-labeled probes in dot and Southern hybridizations at high stringency, four of five probes consistently hybridized to DNA extracts derived from LY-affected palms only. At moderate stringency, all probes hybridized with DNA of other MLOs that occur in Florida, and three probes also hybridized to DNA of several *Acholeplasma* and/or *Spiroplasma* species. In addition to Manila palms, probes detected the presence of LY MLO DNA in DNA samples extracted from heart tissues of LY-diseased true date (*Phoenix dactylifera*), cliff date (*P. rupicola*), Chinese fan (*Livistona chinensis*), and five coconut (*Cocos nucifera*) palm cultivars. Probes also hybridized to DNA from symptomatic *Caryota rumphiana* and *L. rotundifolia*, two palm species previously not known to be affected by LY disease.

Lethal yellowing (LY) is a fast-spreading, highly destructive, mycoplasmalike organism (MLO)-associated disease of the coconut palm (*Cocos nucifera* L.) (16,37). The disease presently occurs in several countries of the northern Caribbean region (17,34,38), where it has virtually eliminated the native, tall-type coconut palms from many locales. In Jamaica alone, estimated losses of these palms to LY exceeds 3 million (1). Similar MLO-associated lethal declines of coconut have occurred in West Africa (5,43) and, more recently, in Tanzania, where an estimated 7 million mature palms have been killed (40). Collectively, these diseases pose the most important threat to global coconut production (14).

Epidemic losses of coconut palms to LY in Florida during the 1970s were accompanied by other MLO-associated lethal declines of at least 24 additional palm species (20,44,45). A similarity of symptom expression, coupled with the close chronological and geographic coincidence of the various palm declines with coconut LY, indicated that all of these diseases were probably caused by the same pathogen. Subsequent transmission of the palm lethal yellowing agent to coconut, Manila (*Veitchia merrillii* (Becc.) H. E. Moore), and Thurston (*Pritchardia thurstonii* F. Muell. et Drude) palms by field-collected *Myndus crudus* Van Duzee, the American palm cixiid planthopper and principal suspect vector of LY in the Americas (19), has provided further strong circumstantial evidence that these diseases are synonymous, as was assumed in this study.

A major limitation to acquiring important etiological and epidemiological information concerning LY during the last two decades has been the lack of a rapid, sensitive, and specific means for pathogen detection and disease diagnosis. The LY agent, like other MLOs, is unculturable; consequently, diagnosis of LY disease has relied primarily on symptom identification. Confirmation of diagnoses requires transmission electron microscopy (TEM) examination of phloem tissues for the presence of MLOs, as they are too small to be resolved by light microscopy. Although TEM has provided a means for detection of the LY MLO, false negative diagnoses frequently result from these time-consuming and costly examinations because of the very low concentrations and uneven distribution of MLOs in palms (44,45).

Substantial progress has been made in recent years toward improved detection and diagnosis of MLO-associated diseases by the application of both hybridoma and recombinant DNA technologies to this problem. Monoclonal antibodies against a few MLOs have been developed, and their sensitivity and specificity for detection of these MLOs have been demonstrated (3,4,21,31). Nucleic acid hybridizations using cloned random fragments of MLO DNA as probes also are providing a reliable and accurate means of detecting numerous MLOs (2,6,9,15,23,24,26) and are being used to differentiate MLOs (2,10,27,28) as well as to monitor their distribution and ecology (22).

The purpose of this study was to develop DNA hybridization probes for detection of the MLO associated with lethal yellowing disease of palms. The extraction and cloning of MLO DNA from an LY-diseased Manila palm is described. We characterized and used five cloned DNA probes for detection of the lethal yellowing agent in seven symptomatic palm species.

MATERIALS AND METHODS

Sources of healthy and diseased palms. Palm heart (= meristem) tissue was excised from various naturally infected palms displaying early foliar symptoms indicative of LY disease. Samples were obtained during 1988–1990 from representatives of seven palm species that included *C. nucifera* (cultivars Jamaica Tall, Hawaiian Tall, Samoan Dwarf, Panama Tall, and Malayan Dwarf), true

date (*Phoenix dactylifera* L.) palm, cliff date (*P. rupicola* T. Anderson) palm, *Caryota rumphiana* Mart., Chinese fan (*Livistona chinensis* (Jacq.) R. Br. ex Mart.) palm, *L. rotundifolia* (Lam.) Mart., and Manila (*V. merrillii*) palm. Three 3-yr-old Manila palms were provided from a backyard planting by J. H. Tsai within 3 wk after the appearance of LY symptoms. These palms had been deliberately exposed to *M. crudus* planthoppers, which were collected periodically from neighborhood palms and grasses and then confined to their foliage in sleeve cages. Seventy-five samples each consisting of 20–35 planthoppers were caged on each palm during a 7-mo period.

Additional LY-infected Manila palms and one queen palm (*Syagrus romanzoffianum* (Cham.) Glassman), a species not known to be susceptible to LY but exhibiting an advanced stage of bacterial bud rot, were provided by local homeowners. All other 7- to 10-yr-old diseased palms were taken from the palm collection located on the grounds of the Fort Lauderdale Research and Education Center. LY disease pressure on this collection is purposely maintained at a relatively high level by continuous interplanting with known susceptible palms. Young (1- to 2-yr-old) shadehouse grown palms, rather than symptomless palms from the landscape, provided sources of healthy tissues for experimental controls.

Sources of other mollicutes. Plants affected by various other MLO-associated diseases indigenous to Florida were maintained in screenhouses. These included Aristogold Guardian Evergreen sweet corn (*Zea mays* (L.) 'saccharata') with maize bushy stunt (MBS) (6), periwinkle (*Catharanthus roseus* (L.) G. Don) plants with Florida periwinkle witches'-broom (FWB) (35), and pigeon pea witches'-broom (WBP) (15). Eastern aster yellows (EAY) in periwinkle was provided by J. A. Wyman, University of Wisconsin, Madison.

Spiroplasma kunkelii (SK) Florida isolate M1 (7); *S. citri* (SC) California isolate 189 (33); the *Cocos* spiroplasma (CS) isolate N525 (13) provided by R. Whitcomb, USDA, ARS, Beltsville, MD; *S. apis* isolate PPS-1 (36); *S. floricola* isolate 23-6 (8); and *Acholeplasma florum* isolate GF1 (36) were cultured in C3-G (29) medium. *A. oculi* (19L), *A. axanthum* (S743), and an as yet unidentified *Acholeplasma* sp. (J233) from coconut palm (12), provided by J. G. Tully, Frederick Cancer Research Facility, Frederick, MD, were cultured in SP-4 medium (46).

DNA extractions. Petiole bases of unemerged leaves were excised within 2 h of harvest from 100 to 200 g of palm heart tissues. These tissues first were rinsed with tap water, blotted dry with paper toweling, and then briefly ground with a Waring blender in ice-cold PS (23) 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, 2.0% polyvinylpyrrolidone [PVP-40], 30 mM ascorbic acid, 10 mM EDTA), using 4 ml of buffer per gram of tissue. The resulting brei was strained through two layers of cheesecloth. After differential centrifugation of 200-ml aliquots of filtrate to remove intact starch and chloroplasts, DNA was extracted from the MLO-enriched pellets by the method of Dellaporta et al (11). Each pellet derived from 50 g of tissue was resuspended in 15 ml of DNA extraction buffer (100 mM Tris-HCl, pH 8, 50 mM disodium EDTA, pH 8, 500 mM NaCl,

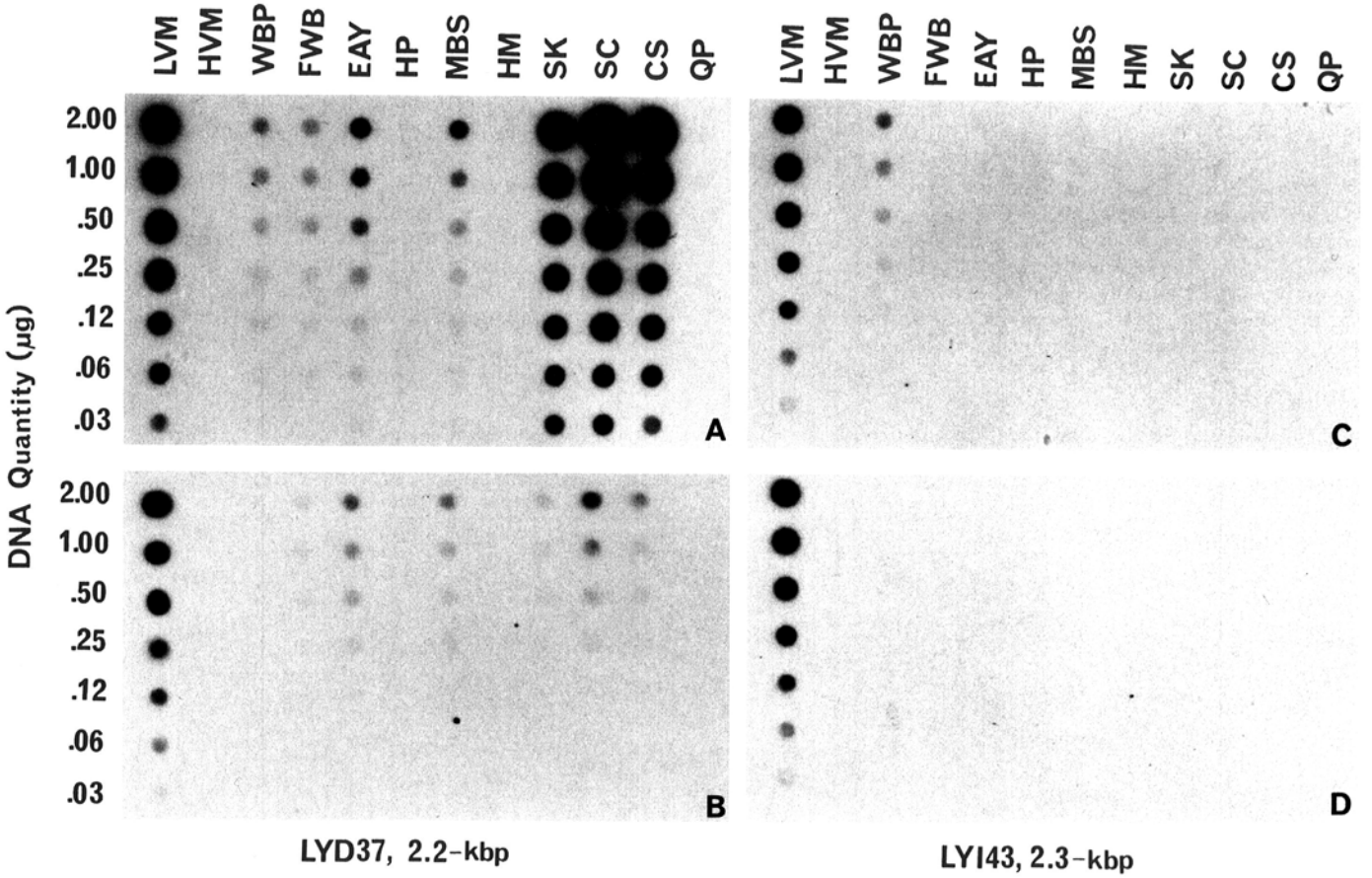

Fig. 1. Dot hybridizations of ^{32}P-labeled cloned lethal yellowing (LY) mycoplasmalike organism (MLO) DNA to DNA extracted from healthy plants, plants affected by other Florida MLO-associated diseases, and several culturable mollicutes. Replicate filters were screened with **A** and **B,** probe LYD37 (a 2.2-kbp *Eco*RI insert of chromosomal LY MLO DNA), or **C** and **D,** probe LYI43 (a 2.3-kbp *Eco*RI insert of chromosomal LY MLO DNA), followed by moderate- (**A** and **C**) or high- (**B** and **D**) stringency wash conditions. LVM, Manila (*Veitchia merrillii*) palm with lethal yellowing; HVM, healthy Manila palm; WPB, periwinkle (*Catharanthus roseus*) with pigeon pea witches'-broom; FWB, periwinkle with Florida periwinkle witches'-broom; EAY, Eastern aster yellows in periwinkle; HP, healthy periwinkle; MBS, maize bushy stunt-infected corn (*Zea mays* 'saccharata'); HM, healthy corn; SK, *Spiroplasma kunkelii*; SC, *S. citri*; CS, *Cocos* spiroplasma; QP, queen palm (*Syagrus romanzoffianum*) with bacterial bud rot.

and 10 mM mercaptoethanol) to which 1 ml of 20% sodium dodecyl sulfate was added. The mixture was incubated at 65 C for 15 min with periodic gentle agitation. After addition of 5 ml of 5 M potassium acetate to the lysate, the mixture was chilled on ice for 30 min and then centrifuged at 25,000 *g* for 20 min at 4 C in a Beckman JA 20 rotor. Nucleic acids were precipitated from the aqueous supernatant by addition of a 0.6 vol of cold isopropanol.

Nucleic acids were collected by centrifugation at 20,000 *g* for 15 min, resuspended in 3 vol of 50 mM Tris-HCl (pH 8) and 20 mM EDTA, and then extracted twice with equal volumes of Tris-neutralized phenol/chloroform/isoamyl alcohol (25:24:1) and once with chloroform/isoamyl alcohol (24:1) alone (32). Nucleic acids were precipitated again by addition of a 0.1 vol of 3 M sodium acetate (pH 5.2) and cold isopropanol, then collected by centrifugation as described previously. Upon resuspension in TE (10 mM Tris-HCl, pH 8, 1 mM EDTA, pH 8) buffer containing 50 μg of RNAse per milliliter, nucleic acid solutions were incubated at 37 C for 1 h and stored at 4 C. DNA from 25-g quantities of healthy corn (stem tissues) or corn with MBS, or healthy periwinkle (shoots) and periwinkle with either WBP, FWB, or EAY also was extracted in a similar manner.

Cells from spiroplasma and acholeplasma cultures were harvested by centrifugation at 20,000 *g* for 30 min at 4 C in a JA 20 rotor. Pellets were resuspended in 100 mM Tris (pH 8.0), 50 mM EDTA, 500 mM NaCl buffer containing 1% SDS, and DNA was extracted as above. Total DNA also was extracted from 5 g of bacterial bud rot-affected queen palm heart tissues by the procedure of Dellaporta et al (11).

Molecular cloning and screening of recombinants. Cesium chloride-bisbenzimide buoyant density gradient centrifugation, which separates A+T rich MLO DNA from mixtures with plant DNA (15,24,41), was used to separate LY MLO DNA from LY-diseased palm DNA preparations. Gradients containing 250–300 μg of DNA from each of three LY-affected Manila palms (provided by J. H. Tsai) and a healthy palm were centrifuged at 227,640 *g* for 24 h in a Beckman JA 65.2 VTi rotor. Banding patterns were examined under long wave UV light. A faintly discernible band of putative MLO DNA that appeared uppermost in one of the LY-diseased Manila palm gradients was used for molecular cloning experiments.

Approximately 900 ng of gradient-enriched MLO DNA was partially digested with the restriction endonuclease *Eco*RI (Boehringer Mannheim Biochemicals, Indianapolis, IN) for 1 h at 37 C. Resulting fragments were ligated with *Eco*RI-digested pUC8 (47) and used to transform *Escherichia coli* DH5α cells (Gibco BRL Life Technologies Inc., Gaithersburg, MD) according to the supplier's instructions.

Recombinants were initially screened by differential colony hybridizations with ^{32}P-labeled, gradient-enriched LY MLO DNA or with total DNA from healthy Manila palm. Probe DNA was labeled by nick translation with [^{32}P]-dATP after the protocol provided by the manufacturer (NEN Research Products, Boston, MA). Colony hybridizations were performed as described by Maniatis et al (32). Selected colonies that hybridized with MLO DNA but produced weak or indiscernible signals with healthy palm DNA were subcultured. Recombinant plasmids were extracted from small scale preparations (minipreps) of each culture by alkaline lysis (32). The size of cloned DNA inserts in recombinant plasmids was determined after restriction endonuclease digestion and electrophoresis in 0.75% Sea Kem GTG agarose (FMC Bioproducts, Rockland, ME) gels with IX TAE (40 mM Tris-acetate, 1 mM EDTA, pH 7.0) buffer. Plasmid DNA digests were blotted from gels onto nylon membranes (Nytran, Schleicher and Schuell Inc., Keene, NH) by the method of Southern (42) using 10× SSC (1× SSC = 150 mM NaCl, 15 mM sodium citrate, pH 7.0) as the transfer buffer. Replicate blots were rinsed with 2× SSC, air-dried, baked at 80 C for 30 min, and hybridized to either ^{32}P-labeled healthy palm DNA or labeled, gradient-enriched LY MLO DNA probes.

Single cloned DNA inserts that hybridized specifically with enriched LY MLO DNA were used as probes. Insert DNA was purified from recombinant plasmid digests by electrophoresis in 0.75% Sea Plaque GTG agarose (FMC Bioproducts) gels and recovered from gel slices by DEAE cellulose chromatography (Elutip-d columns, Schleicher and Schuell) using the procedure of Schmitt and Cohen (39). Each DNA insert (100 ng) was labeled with ^{32}P-dATP by using random oligoprimers (random primed DNA labeling kit, Boehringer Mannheim) and recovered by Sephadex G50 column chromatography as described by Maniatis et al (32).

DNA hybridizations. For dot hybridizations, 2 or 4 μg of DNA extracted from healthy or MLO-infected plants, or from spiroplasma and acholeplasma cultures, was denatured by boiling with NaOH, cooled on ice, and then neutralized by the addition of 2 M Tris, pH 7.0 (15). Samples were blotted onto Nytran membranes as a twofold dilution series (beginning with 1 or 2 μg) by using a Bio-Dot manifold (Bio-Rad Laboratories,

TABLE 1. Summary of results from dot hybridizations of ^{32}P-labeled cloned fragments of lethal yellowing (LY) mycoplasmalike organism (MLO) DNA to DNA extracts from healthy plants, plants affected by various MLO-associated diseases, and culturable mollicutes

Probe[a]	Assay stringency[b]	Hybridization of probe with indicated DNA sample[c]																	
		HVM	HP	HM	LVM	FWB	EAY	WBP	MBS	SK	SC	CS	SF	SA	AF	AA	AO	AU	QP
LYD37	MS	−	−	−	+	W	+	W	+	+	+	+	+	+	+	+	+	+	−
	HS	−	−	−	+	W	W	−	W	−	+	W	−	−	−	W	W	+	−
LYI26	MS	−	−	−	+	+	+	+	+	W	W	−	+	W	W	+	+	W	−
	HS	−	−	−	+	−	−	−	−	−	−	−	−	−	−	−	−	−	−
LYD9	MS	−	−	−	+	W	W	+	W	−	−	−	−	−	NT	−	W	+	−
	HS	−	−	−	+	−	−	−	−	−	−	−	−	−	NT	−	−	−	−
LYI43	MS	−	−	−	+	−	−	+	−	−	−	−	−	−	−	−	−	−	−
	HS	−	−	−	+	−	−	−	−	−	−	−	−	−	−	−	−	−	−
LYD11	MS	−	−	−	+	−	W	W	−	−	−	−	−	−	−	NT	NT	NT	−
	HS	−	−	−	+	−	−	−	−	−	−	−	−	−	−	NT	NT	NT	−

[a] Probes consisted of cloned MLO DNA inserts only.

[b] MS, moderate stringency (55 C), posthybridization wash conditions; HS, high stringency (65 C), posthybridization wash conditions.

[c] +, Positive hybridization signal; W, weak positive signal; −, no signal; NT, not tested. DNA sample from HVM, healthy Manila (*Veitchia merrillii*) palm; HP, healthy periwinkle (*Catharanthus roseus*); HM, healthy corn (*Zea mays* 'saccharata'); LVM, Manila palm with LY; FWB, periwinkle with Florida periwinkle witches'-broom; EAY, periwinkle with Eastern aster yellows; WBP, periwinkle with pigeon pea witches'-broom; MBS, corn infected with maize bushy stunt; SK, *Spiroplasma kunkelii*; SC, *S. citri*; CS, *Cocos* spiroplasma (N525); SF, *S. floricola* (23-6); SA, *S. apis* (PPS1); AF, *Acholeplasma florum* (GF1); AA, *A. axanthum* (S743); AO, *A. oculi* (19L); Au, unknown *Acholeplasma* sp. (J233) from coconut palm; QP, queen palm (*Syagrus romanzoffianum*) with bacterial bud rot.

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 h.

Membranes were prehybridized at 68 C for 16 h in 6× SSC, pH 7.0, containing 10× Denhardt's solution (0.1% Ficoll, 0.1% polyvinylpyrrolidone, 0.1% bovine serum albumin), 0.5% SDS, and 1 mg/ml denatured salmon sperm DNA. Blots were hybridized to probes at 68 C for 16 h in the same solution and 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 then were sealed in plastic wrap and exposed to X-Omat AR diagnostic film (Eastman Kodak, Rochester, NY) with an intensifier screen (Lightning Plus, Du Pont, Newark, DE) for a minimum of 16 h at −75 C. Following autoradiography, membranes were rewashed at increased stringency (65 C) and exposed again to diagnostic film.

For Southern hybridizations, 2 μg of undigested DNA from healthy and from various MLO-infected plant hosts were electrophoresed in 0.75% Sea Kem GTG agarose gels. DNA profiles in gels were blotted onto Nytran membranes and probed with ^{32}P-labeled cloned insert DNA as before.

RESULTS

DNA isolation. Density gradient centrifugation of DNA from healthy Manila palm resulted in the separation of one major and two minor bands. The major band with a buoyant density (ρ) of approximately 1.628 g/cm^3 was located between 28 and 34 mm, and the two minor bands at 36 (ρ = 1.640 g/cm^3) and 38 mm (ρ = 1.656 g/cm^3) from the bottom of the 52-mm gradient tube, respectively. In gradients containing DNA from LY-diseased Manila palms, an additional faintly discernible band, positioned at 8.5 mm above the main host DNA band, was evident. This unique band (ρ = 1.592 g/cm^3) of putative MLO DNA was

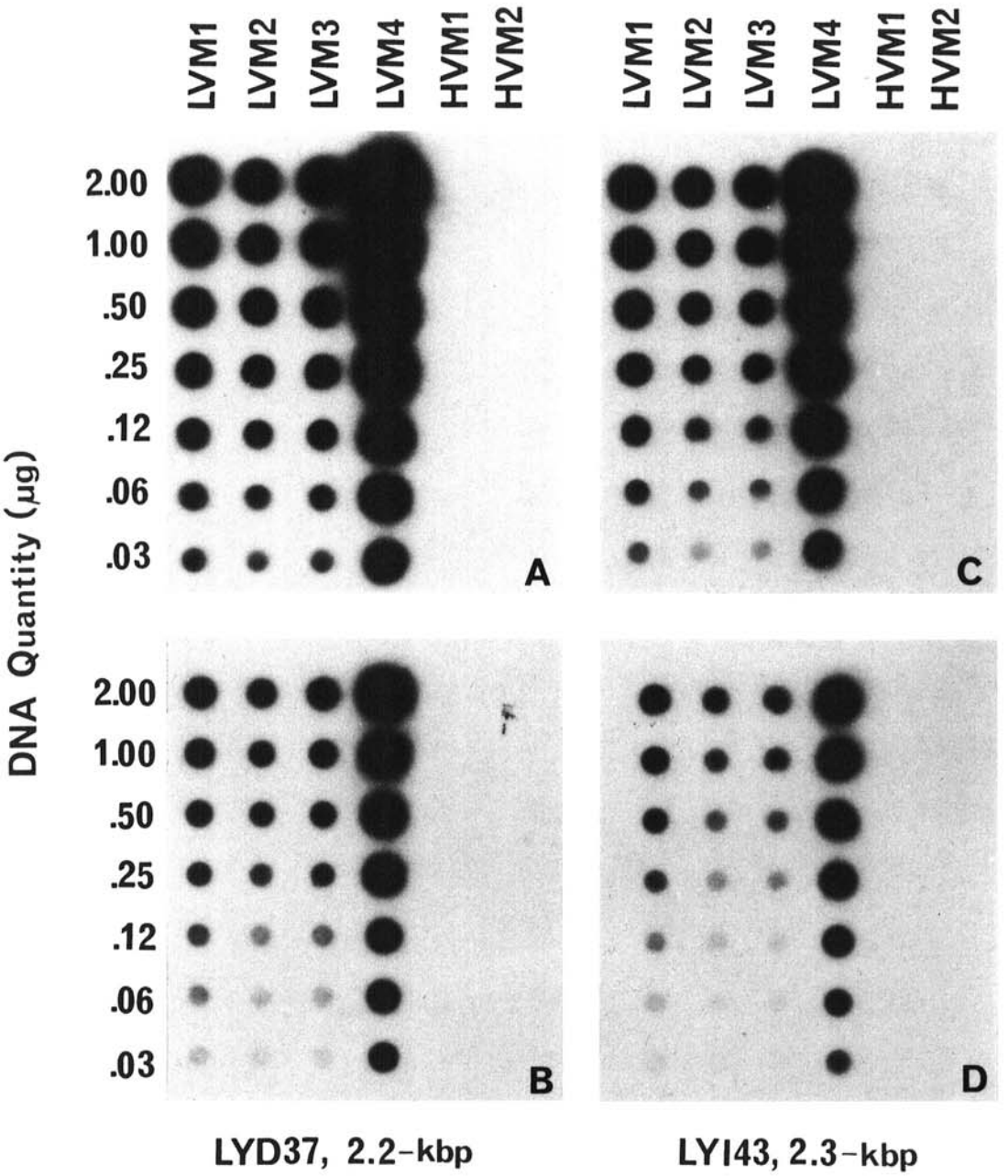

Fig. 2. Dot hybridizations of ^{32}P-labeled cloned *Eco*RI fragments of lethal yellowing (LY) mycoplasmalike organism (MLO) DNA to DNA extracted from healthy and LY-diseased Manila (*Veitchia merrillii*) palms. Replicate filters were screened with **A** and **B,** probe LYD37 (a 2.2-kbp insert of chromosomal LY MLO DNA), or **C** and **D,** probe LYI43 (a 2.3-kbp insert of chromosomal LY MLO DNA), followed by moderate- (**A** and **C**) or high- (**B** and **D**) stringency wash conditions. DNA from LVM1–4, Manila palms with lethal yellowing, and HVM1–2, healthy Manila palms.

removed and used for molecular cloning purposes. About 1.2 μg of enriched MLO DNA was recovered and represented about 0.4% of the total DNA loaded to the initial gradient. Similar fractionation of each remaining gradient of diseased palm DNA yielded between 100 and 200 ng of DNA although no band was evident. A small quantity of DNA (~20 ng) also was recovered from this region of the gradient containing healthy host DNA.

A total of 880 recombinant colonies were obtained in the cloning of enriched LY MLO DNA from Manila palm. Of the 710 colonies initially screened by differential colony hybridizations with ^{32}P-labeled total DNA from healthy Manila palm and with gradient-enriched LY MLO DNA, 102 colonies hybridized to the MLO DNA probe but hybridized only weakly, or not at all, with healthy host DNA. Similarly, DNA inserts from 22 of 81 recombinant plasmids that were investigated further hybridized strongly to enriched LY MLO DNA probe only. Twelve of these represented single cloned DNA inserts. When each excised insert was used as probe in preliminary dot hybridizations, five inserts of unique size ranging from 4.1 to 1.2 kbp hybridized to DNA from LY-diseased Manila palm and not to DNA from healthy palms.

Characterization of probes. To determine the specificity of the five disease-specific, cloned inserts for detecting LY MLO DNA, each was used as probe in dot hybridizations with DNAs from other MLOs indigenous to Florida and with DNAs of several *Acholeplasma* and *Spiroplasma* species. A summary of results from once-repeated hybridizations under both moderate (MS) and high stringency (HS) conditions is given in Table 1. No hybridizations occurred between any probe and DNAs from healthy host plants that included Manila palm, periwinkle, and corn, regardless of assay stringency. Probes hybridized to varying extents with remaining test DNAs and could be grouped into two general categories on the basis of results at moderate stringency. For example, probes LYD37 (2.2 kbp), LYI26 (3.1 kbp), and LYD9 (4.1 kbp) hybridized broadly to DNA of Florida MLOs as well as to acholeplasma and/or spiroplasma DNAs. Characteristically, probe LYD37 hybridized to DNA of the *Cocos*

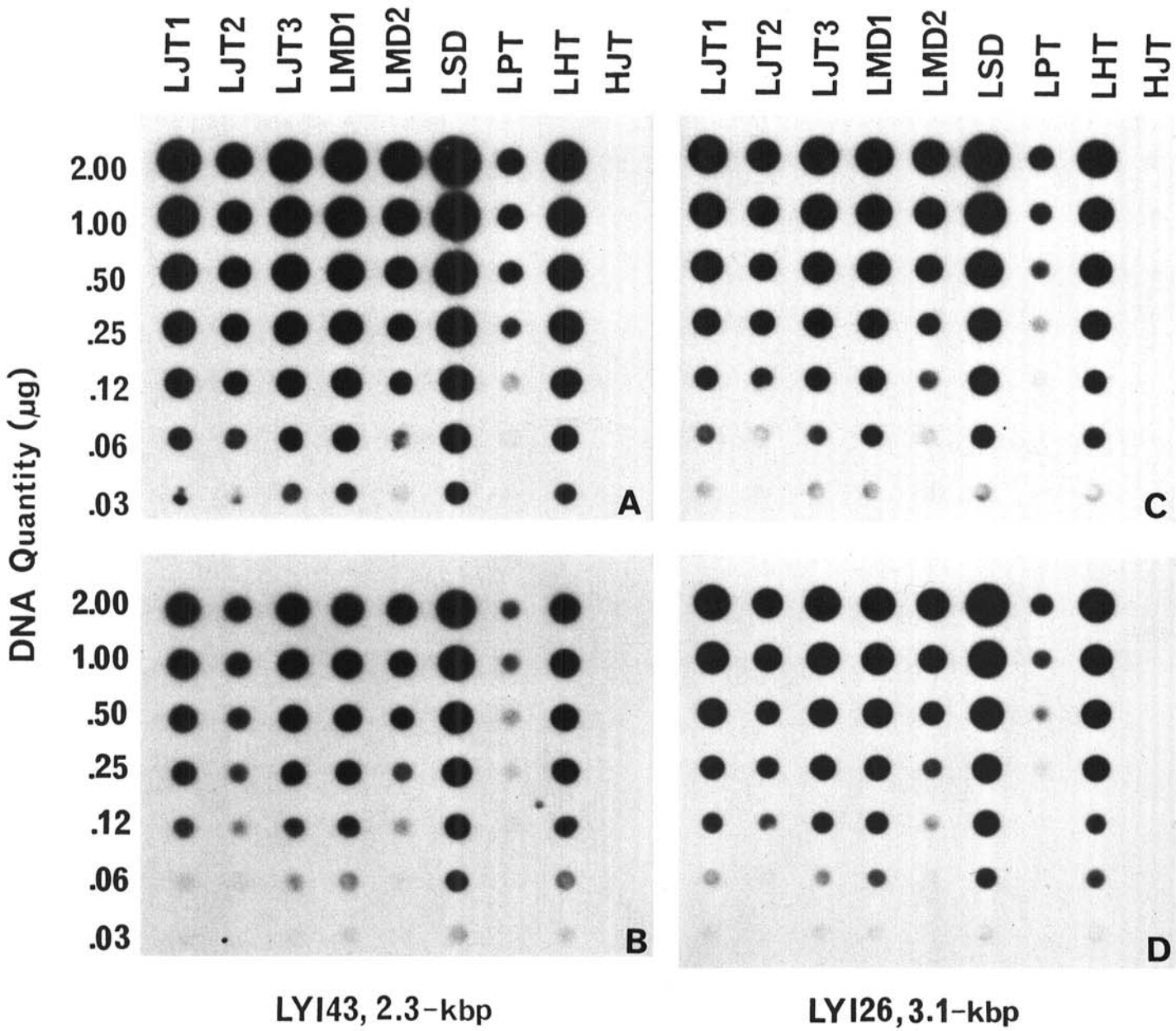

Fig. 3. Dot hybridizations of ^{32}P-labeled cloned *Eco*RI fragments of lethal yellowing (LY) mycoplasmalike organism (MLO) DNA to DNA extracted from five LY-affected coconut (*Cocos nucifera*) palm cultivars and from healthy palms. Replicate filters were screened with **A** and **B**, probe LYI43 (a 2.3-kbp insert of chromosomal LY MLO DNA), or **C** and **D**, probe LYI26 (a 3.1-kbp insert of chromosomal LY MLO DNA), followed by moderate- (**A** and **C**) or high- (**B** and **D**) stringency wash conditions. DNA from LY-affected coconut palm cultivars LJT1–3, Jamaica Tall; LYMD1–2, Malayan Dwarf; LSD, Samoan Dwarf; LPT, Panama Tall; and LHT, Hawaiian Tall. HJT, DNA from healthy Jamaica Tall.

spiroplasma, whereas probe LYI26 did not. By comparison, probe LYD9 hybridized to acholeplasma but not to spiroplasma DNAs. The second category consisted of LYI43 (2.3 kbp) and LYD11 (1.2 kbp), two probes that hybridized weakly to various MLO DNAs, but not to DNA of culturable mollicutes. Results representative of hybridizations between a probe from each moderate stringency category, namely, LYD37 and LYI43, to various MLO DNAs and to DNAs of *S. kunkelii, S. citri,* and the *Cocos* spiroplasma, are illustrated in Figure 1A and C. Hybridization signals as a result of screening DNAs of *S. apis, S. floricola, A. florum, A. axanthum, A. oculi,* and an unidentified *Acholeplasma* sp. with probe LYD37 were comparable with those observed for this probe and other spiroplasma DNAs. Also, signals resulting from hybridizations of probes LYI26 and LYD9 to mollicute DNAs were consistently weaker than those observed for LYD37 (data not shown). When results of hybridizations between each of the five probes and test DNAs were reevaluated at higher stringency, four probes including LYI43 (Fig. 1D) hybridized only to DNA from LY-diseased Manila palm. However, faint but discernible hybridization signals remained for

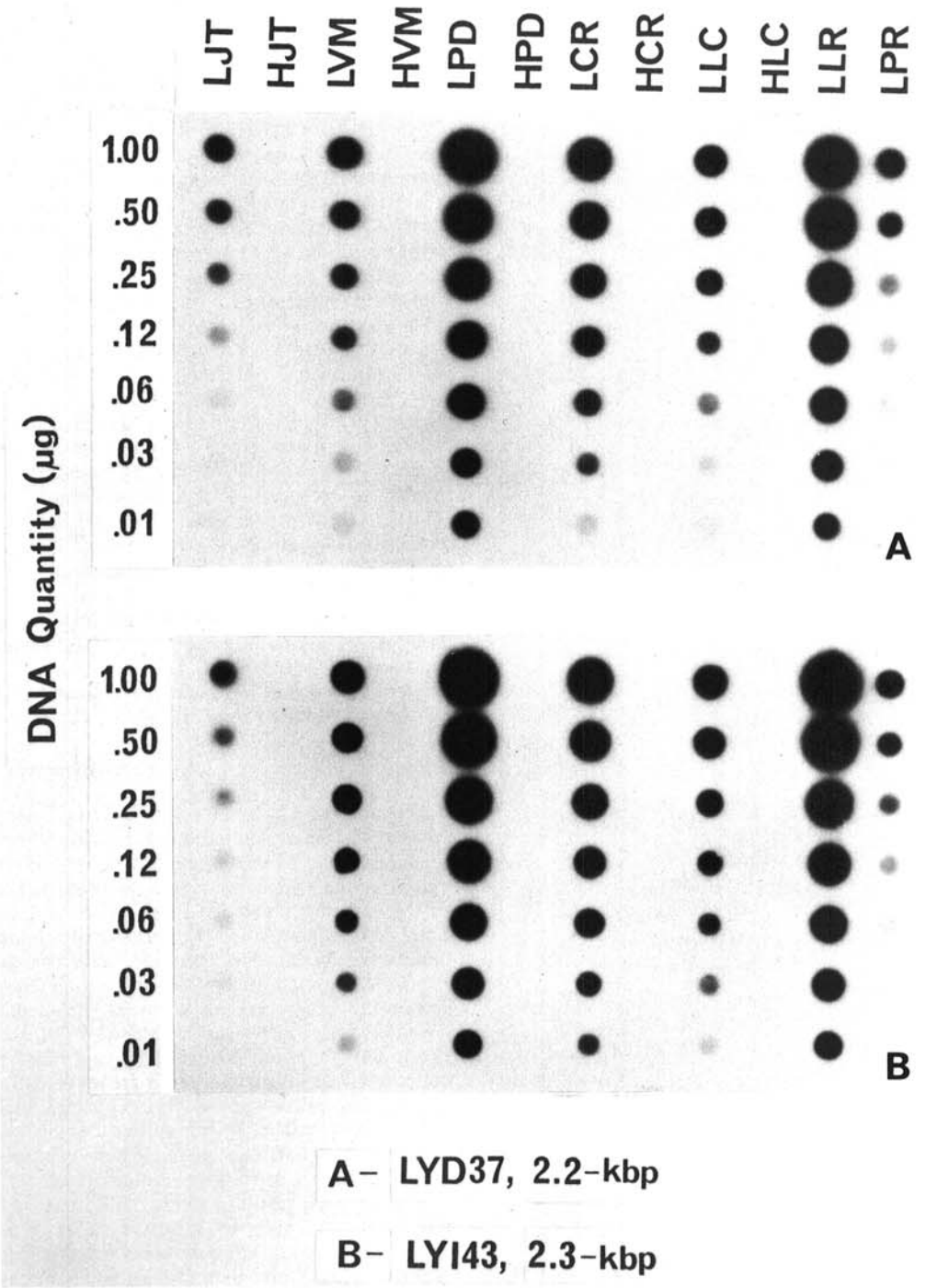

Fig. 4. Detection of lethal yellowing (LY) mycoplasmalike organism (MLO) DNA in DNA extracted from heart tissues of healthy and LY-affected palm species by dot hybridization assays using cloned *Eco*RI fragments of LY MLO DNA as probes. Replicate blots were screened with ^{32}P-labeled DNA probes **A,** LYD37 (a 2.2-kbp insert of chromosomal LY MLO DNA), or **B,** LYI43 (a 2.3-kbp insert of chromosomal LY MLO DNA), and subjected to high-stringency wash conditions. DNA from LJT, LY-diseased coconut (*Cocos nucifera*) cv. Jamaica Tall; HJT, healthy Jamaica Tall; LVM, LY-diseased Manila (*Veitchia merrillii*) palm; HVM, healthy Manila palm; LPD, LY-diseased true date (*Phoenix dactylifera*) palm; HPD, healthy date palm; LCR, LY-diseased *Caryota rumphiana* palm; HCR, healthy *C. rumphiana;* LLC, LY-diseased Chinese fan (*Livistona chinensis*) palm; HLC, healthy Chinese fan palm; LLR, LY-diseased *L. rotundifolia;* LPR, LY-diseased cliff date (*P. rupicola*) palm.

most MLO and mollicute DNAs probed with LYD37 (Fig. 1B).

Each cloned probe was used in Southern hybridizations with undigested DNA extracts from healthy plants and with DNA from MLO-enriched extracts derived from plants affected by various MLO-associated diseases. Patterns of hybridization between probes and each DNA profile at high stringency were quite similar. All probes hybridized strongly to DNA from LY-diseased Manila palm on blots at a location corresponding to the position of the visible high molecular weight chromosomal DNA band in the adjacent gel. By comparison, no discernible hybridizations were observed between probes and other DNAs. However, at moderate stringency, faint signals were evident from chromosomal DNA bands of DNA profiles from other MLO-infected plants (data not shown). The Southern hybridization patterns observed between each probe and test DNA combination at reduced stringency were the same as those recorded for dot blots (Table 1).

Detection of the LY MLO in diseased palms. Dot hybridizations were used to assess the utility of probes for detecting the LY MLO in symptomatic palms. Probes first were used to screen DNA samples obtained from both healthy and diseased Manila and coconut palms. The stringency of hybridizations was varied, as before. Identical patterns of hybridization were evident between each of the five probes and the various Manila or coconut DNA samples. Typical signals resulting from screening Manila palm DNAs with probes LYD37 or LYI43, and coconut palm DNAs with probes LYI43 and LYI26, are illustrated in Figures 2 and 3, respectively. Neither probe LYD37 nor LYI43 hybridized to healthy Manila palm DNA at either moderate (Fig. 2A and C) or high stringency (Fig. 2B and D). Similarly, neither LYI43 nor LYI26 hybridized to healthy coconut palm DNA at either moderate (Fig. 3A and C) or high stringency (Fig. 3B and D). By contrast, strong hybridizations occurred between probes and DNAs extracted from all diseased Manila (Fig. 2) and coconut (Fig. 3) palms, regardless of stringency conditions.

In dot hybridization assays, probes routinely detected the presence of LY MLO DNA in 2 μg of palm DNA extract and, in several instances, in as little as 30 ng of extract. However, lower limits of detection of MLO DNA in DNA samples from the same LY-affected palm species varied according to the particular DNA sample tested (e.g., Fig. 2A or 3A) and with the stringency conditions of the hybridization assay. Two- or fourfold reductions in probe detection sensitivity resulted when hybridizations were performed at high rather than moderate stringency (e.g., Fig. 2C compared with D).

Selected probes were subsequently used in a series of dot hybridizations with DNAs from five LY-affected palm species in addition to Manila and coconut palms. Results representative of hybridizations obtained with probes LYD37 and LYI43 at high stringency are shown in Figure 4A and B, respectively. Neither probe hybridized to DNAs from healthy host palms. Both probes hybridized to DNA from all seven diseased palm species including *L. rotundifolia* and *C. rumphiana,* two species not previously recorded as LY-susceptible hosts. Also, the presence of MLO DNA was detected by probes in six- to eightfold lower concentrations of DNA extracts from LY-diseased *P. dactylifera* (LPD) and *L. rotundifolia* as compared with extracts from *C. nucifera* 'Jamaica Tall' (LJT) or *P. rupicola* (LPR).

At moderate stringency, probe LYI43 hybridized to similar multiple restriction fragments in *Eco*RI digests of twice gradient-enriched LY MLO DNA from diseased Chinese fan palm (*L. chinensis*) as well as DNA derived from an MLO-enriched extract of diseased Manila palm (Fig. 5).

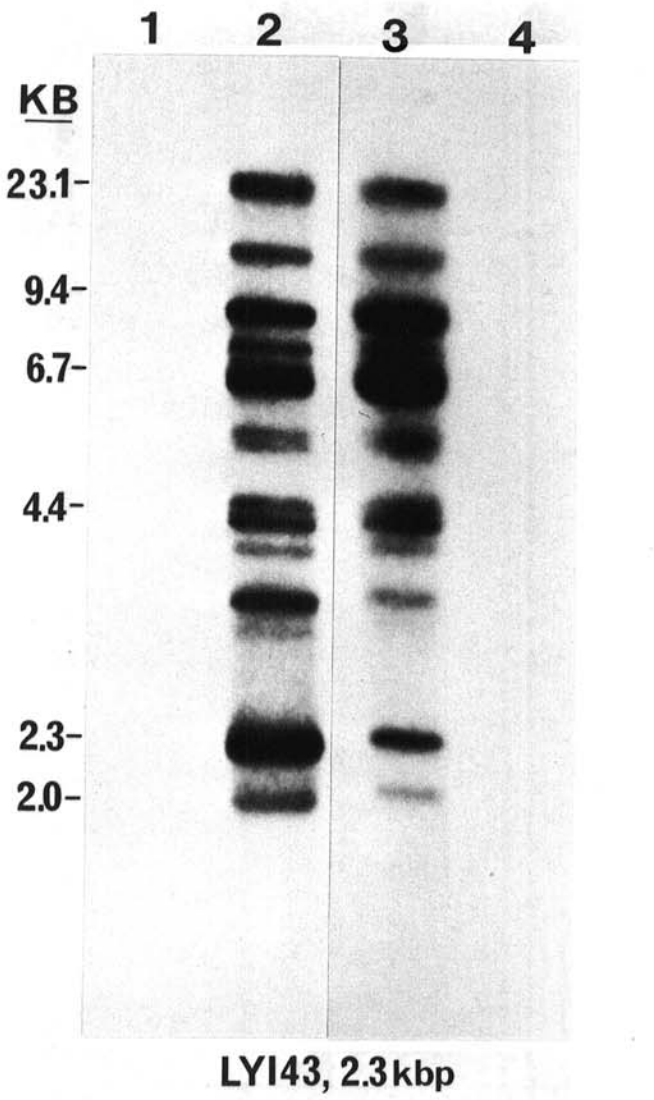

Fig. 5. Southern blot analysis of *Eco*RI-digested DNA extracts from healthy and lethal yellowing (LY)-diseased palms hybridized to ^{32}P-labeled cloned DNA probe LYI43 (a 2.3-kbp *Eco*RI insert of chromsomal LY mycoplasmalike organism DNA). The blot was subjected to moderate-stringency wash conditions. 1, total DNA from healthy Chinese fan (*Livistona chinensis*) palm; 2, CsCl-bisbenzimide density gradient-enriched LY MLO DNA from Chinese fan palm; 3, total DNA from LY-diseased Manila (*Veitchia merrillii*) palm; 4, total DNA from healthy Manila palm.

DISCUSSION

Because the LY agent rarely is found in most mature palm tissues (44,45), we limited our extraction attempts to immature, unemerged leaf bases of the crowns of palms where the pathogen can be found with reasonable regularity, although less than 5% of the vascular bundles of these tissues typically contain MLOs (45). To compensate for the anticipated low numbers of MLOs, comparatively large amounts of these tissues, typically entire hearts, were extracted. With the exception of the resistant Malayan Dwarf, all other coconut cultivars included in this study are considered to be highly susceptible to LY (18) and as such provided the most frequent source of samples. However, MLO concentrations in coconut palms are reportedly among the lowest of any susceptible species (45). This previous conclusion was further substantiated by our earlier inability to resolve an MLO DNA band on CsCl-bisbenzimide density gradient centrifugation of DNA samples derived from these palms.

Experimental transmission of LY disease to Manila, coconut, and Thurston palms by exposure to *M. crudus* planthoppers collected from LY-affected areas of south Florida has been reported (19). Coincidentally, young landscape Manila palms exposed to supplemental, field-collected *M. crudus* in this study also contracted the disease. MLO titers in this moderately susceptible species (18) were subsequently found to be quite variable. However, tissues from one of these palms apparently contained a sufficient quantity of the LY MLO to enable separation of a detectable band of MLO-associated DNA from the ensuing DNA extract. When similar DNA extracts were

prepared from additional palm species, those species exhibiting the strongest hybridization signals also were found to contain sufficient MLO DNA such that this DNA could be resolved by density gradient separations.

Detection of mollicute DNA by probes varied according to the particular probe and test DNA extract and may reflect any combination of several factors, including the concentration of mollicute DNA present in sample extracts, the degree of base sequence similarity between each probe and test DNA, the copy number of cloned sequences, probe size, and hybridization stringency. Signals observed after screening samples of spiroplasma DNA with probe LYD37 were as strong, or stronger than, signals produced by this probe and DNA from LY-diseased Manila palm at moderate stringency (Fig. 1A). This was attributed mostly to reflect the quantity of detectable DNA present in each extract. DNA from mollicute cultures consisted of 100% mollicute DNA, whereas less than 0.4% of the equivalent quantity of Manila palm DNA sample actually consisted of LY MLO DNA, as determined by earlier density gradient analyses. Thus, a comparison of hybridization signals as a result of screening 8 ng of each spiroplasma DNA with those obtained for 2 μg of LY-diseased Manila palm DNA would have provided, perhaps, a more meaningful evaluation of relative signal differences between these test DNA samples. The overall sensitivity of the various cloned probes to detect LY MLO DNA in all of the DNA samples from diseased palms may be explained, in part, by the observation that probe LYI43 hybridized to multiple fragments in *Eco*RI digests of two of these DNAs.

Necrosis of the spear leaf and progressive soft rot of heart tissues are typical of the terminal phase of the LY disease syndrome in most palms (12,44). Both *Acholeplasma* spp. (predominantly *A. axanthum* and *A. oculi*) (12) and the *Cocos* spiroplasma (13) were isolated from decaying heart and inflorescence tissues, respectively, on LY-affected coconut palms in Jamaica. Thus, necrotic tissues and these potential contaminants were avoided by harvesting heart samples shortly after LY symptoms appeared. Nevertheless, we also included total DNA extracts from necrotic queen palm tissue as a specificity check. Since this palm is not susceptible to LY, any hybridizations between probes and this DNA, had they occurred, would have indicated that the probes may have been derived from DNA of microorganisms other than the LY agent commonly found in or on such tissues.

To date, there is only one report of hybridization between a cloned fragment of MLO-associated DNA and DNA of a culturable mollicute (6). This is somewhat surprising since a recent phylogenetic analysis of 16S rRNA sequences of the *Oenothera* MLO concluded that these MLO gene sequences most closely resemble those of acholeplasmas (30). The initial finding that two of our probes, namely LYD37 and LYI26, hybridized at moderate stringency to DNA of *S. kunkelii,* the corn stunt pathogen, was the principal reason we expanded probe characterization to include hybridizations with additional spiroplasma and acholeplasma DNAs. Although both probes share sufficient nucleotide sequence similarity to hybridize with DNA of several other mollicutes at moderate stringency, the reduction or elimination of hybridizations between probes and test DNAs at high stringency demonstrates their LY disease specificity. However, lack of specificity of each of the probes at moderate stringency requires that hybridizations be performed at high stringency to discriminate the presence of LY MLO DNA from other mollicute DNAs.

While presence of extrachromosomal DNAs, apparently plasmids (25), in MLOs is a trait shared by many of these pathogens, particularly those that induce virescence and/or phyllody symptoms in their respective plant hosts (26), we found no evidence to indicate that the LY MLO contains extrachromosomal DNA or that we cloned such DNA. Apparently, other MLOs, which like the LY agent are implicated as the cause of decline-type diseases of woody perennial plant hosts, also do not contain extrachromosomal DNA (27).

On the basis of accumulated circumstantial evidence and partly for convenience, all MLO-associated lethal declines of palms that occur in Florida are presumed to be caused by the same pathogen (18). Thus, these declines are collectively referred to as LY. The demonstrated homology between cloned probes and diseased palm DNAs in this study supports the contention that MLOs associated with different palm hosts in Florida are quite similar. However, more detailed studies will be required to determine the extent of this similarity.

The utility of the cloned probes for diagnosis of LY disease of Florida palms is clearly demonstrated by our ability to detect the LY MLO DNA in samples from two additional species of palms for the first time. This extends the known host range of LY and increases our understanding of the epidemiology of the disease in the state. The probes may prove useful in the identification of primary and alternate plant hosts and insect vector(s) as both the probes and the disease are further characterized.

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