Molecular Plant Pathology

A Genotype-Based System for Identification and Classification of Mycoplasmalike Organisms (MLOs) in the Aster Yellows MLO Strain Cluster

I.-M. Lee, R. E. Davis, T.-A. Chen, L. N. Chiykowski, J. Fletcher, C. Hiruki, and D. A. Schaff

First, second, and seventh authors: USDA-ARS, MPPL, Beltsville, MD 20705; third author: Department of Plant Pathology, Cook College, Rutgers University, New Brunswick, NJ 08903; fourth author: Plant Research Center, Agriculture Canada, Ottawa Ontario, Canada K1A 0C6; fifth author: Department of Plant Pathology, Oklahoma State University, Stillwater, OK 74078; sixth author: Department of Plant Science, University of Alberta, Edmonton Alberta, Canada T6G 2P5.

Present address of D. A. Schaff: Department of Plant and Soil Science, University of Delaware, Newark 19717.

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DNA probes used in this study are available (contact I.-M. Lee or R. E. Davis).

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ABSTRACT

Lee, I.-M., Davis, R. E., Chen, T.-A., Chiykowski, L. N., Fletcher, J., Hiruki, C., and Schaff, D. A. 1992. A genotype-based system for identification and classification of mycoplasmalike organisms (MLOs) in the aster yellows MLO strain cluster. Phytopathology 82:977-986.

Fifteen mycoplasmalike organism (MLO) strains from North America and Europe, including aster yellows (AY), tomato big bud (BB), clover phyllody (CPh), chrysanthemum yellows (CY), and unknown MLOs, were studied. These MLOs are among those previously identified, on the basis of dot hybridizations, as members of the AY MLO strain cluster. Restriction fragment length polymorphism (RFLP) analyses revealed that all 15 strains can be classified into three genomic types: type I (typified by eastern AY MLOs), type II (e.g., western AY and CY MLOs), and type III (e.g., CPh MLO). Four key DNA probes (pAY9N, pBB88, pBB101, and pCN1-25) were designated for use in RFLP analyses for the identification of strains in the AY MLO strain cluster and the differentiation of the types within this cluster. This classification is consistent with results from monoclonal antibody typing and polymerase chain reaction analysis, but not with classifications based solely on biological properties.

Additional keywords: DNA hybridization, molecular cloning, mollicutes, phylogenetics.

Aster yellows (AY), first studied by Kunkel in New York in 1926 (30), is now believed to be caused by uncultured mycoplasmalike organisms (MLOs) (13,25). Kunkel had experimentally transmitted this AY MLO (then called AY virus) by using the aster leafhopper, *Macrosteles fascifrons* (Stål), on numerous species of plants, but not on some species, including celery (*Apium graveolens* L.) and *Zinnia elegans* Jacq. (30,31). The existence of two AY "virus" strains was first suspected by Severin in 1929, who reported that in California AY virus was readily transmitted to *Zinnia* and celery plants (57). Later, on the basis of symptomotology on differential host plants, Kunkel (32) confirmed that the California strain of AY virus was distinct from the eastern strain. The two strains were designated the western (California) and eastern (New York) strains of AY virus. Generally, the western strain of AY virus was referred to as a celery-infecting strain, and the eastern strain as a non-celery-infecting strain.

Identification and classification of AY virus or AY MLO strains in the following decades were based primarily on vector relationships and symptomatological characteristics of eastern and western strains of the AY pathogen. Many strains collected from various states and from Canada were identified and designated eastern or western AY virus or AY MLO accordingly (5,18,19,21). However, there were strains that shared common vector(s) and/or host plants with the two known strains but induced symptoms in the host plants unlike those caused by either eastern or western

strains of the AY pathogen. Thus, more new strains were proposed (5,44,47,52,53,59). However, as AY or suspected AY diseases were subsequently found in other geographic regions outside North America and as the vector species and host plants varied in different geographic regions, the difficulty in identifying strains among the presumed AY MLO complex was compounded. Few investigators actually conducted identification procedures similar to Kunkel's, and a large number of yellows diseases that may, in fact, be caused by MLO strains closely related to AY MLO were named as new diseases without pathogen identification (47,52). Lack of accurate and reliable means for identification of "non-AY" MLOs and of strains that make up the AY MLO complex undoubtedly poses major problems in phytosanitary regulations and international exchange of germ plasm.

Recently, the advent of recombinant DNA technology has made it possible to clone MLO-specific DNA fragments from infected plants or insect vectors (8–12,4,23,24,28,29,35,38–41,43,50,56). The use of cloned MLO DNA sequences as molecular probes in nucleic acid hybridizations permits reliable and specific detection and identification of MLOs in infected plant or insect tissues. This approach also permits investigation of the genetic relatedness among MLOs from various sources. By employing DNA probes derived from these MLO-specific DNA fragments in dot hybridizations, we previously demonstrated that not only the known eastern and western strains of AY MLO but also other MLOs associated with yellows diseases under designated names other than AY were genetically closely related to one another (9–11,35,38,39). The AY MLO strain cluster that makes up MLO strains sharing extensive sequence homology was proposed (35). Strains in the AY MLO strain cluster are genetically distinct from other MLOs (9,35,39).

The objectives of this study were to further identify strains that might belong to the AY MLO strain cluster and to establish a genotype-based classification scheme for identification of MLO strains in the AY MLO strain cluster. We undertook the present studies first to identify strains that might belong to the AY MLO strain cluster by doing extensive dot hybridizations with DNA probes cloned from Maryland AY MLO (35), tomato big bud MLO (39), and periwinkle little leaf MLO (9). Then, finer analyses on the genetic interrelationships among strains in the AY MLO strain cluster were conducted by Southern hybridization and restriction fragment length polymorphism (RFLP) analyses with these cloned DNA probes. This work has resulted in the reclassification of some previously designated AY strains and the identification of some MLO strains that were designated non-AY MLOs as members of the AY MLO strain cluster. Abstracts of part of this work have been published (36,37).

MATERIALS AND METHODS

Sources of healthy and diseased plants. Diseased plants of periwinkle (*Catharanthus roseus* (L.) G. Don) were maintained by grafting in a white flowered clone of periwinkle. Maryland aster yellows (AY1) (35) was originally field-collected at Beltsville, MD. Clover proliferation (CP) (14), potato witches'-broom (PWB), a strain of Alberta (14) aster yellows (AY27), and a strain of New York (14) aster yellows (NYAY) MLO-infected periwinkle plants were provided by C. Hiruki. Strains of Oklahoma (17) aster yellows (OKAY3, OKAY1) MLO-infected periwinkle plants were provided by J. Fletcher. New Jersey strain (45) of aster yellows (NJAY) MLO-infected periwinkle plants were provided by T.-A. Chen. An eastern strain of aster yellows (NAY) (5) MLO-infected China aster plants, and clover phyllody (CPh) (4) and Canada peach X (CX) MLO-infected periwinkle plants were provided by L. N. Chiykowski. Other MLO strains were kindly provided by the following researchers, who provided each strain separately in periwinkle tissue unless noted otherwise: severe aster yellows (SAY2) (57), dwarf aster yellows (DAY) (57), and Tulelake aster yellows (TLAY2) (57) (A. H. Purcell, University of California, Berkeley); tomato big bud (BB) (7) (J. Dale, University of Arkansas, Fayetteville); periwinkle little leaf (CN1 = Orchard-1, CN13 = Orchard-13) (9) (S. M. Douglas, Connecticut Agricultural Experiment Station, New Haven); ash yellows (AshY) and elm yellows (EY) (W. A. Sinclair, Cornell University, Ithaca, NY); western X-disease (WX) (B. C. Kirkpatrick, University of California, Davis); beet leafhopper-transmitted virescence agent (VR) (G. N. Oldfield, University of California, Riverside); chrysanthemum yellows (CY2) (1) and Italy tomato big bud (BB_I) (A. Bertaccini, Instituto di Patologia Vegetale, Bologna, Italy); Minnesota aster yellows (MNAY) in China aster (E. E. Banttari, University of Minnesota, St. Paul, MN); maize bushy stunt (MBS) in corn (L. R. Nault, Ohio State University, Wooster, OH). Tissue samples of healthy periwinkle (H_V), lettuce (H_L), China aster (H_A), corn (H_C), and tomato (H_T) were provided by our collaborators, accordingly.

Preparation of DNA probes. Eight AY1, eight BB, and six CN1 MLO-specific DNA probes were prepared, respectively, by nick translation of cloned recombinant plasmids (9,35,39) with biotin-7-dATP according to the instructions of the manufacturer (Bethesda Research Laboratories, Gaithersburg, MD). These cloned MLO DNA fragments from AY1, BB, and CN1 MLO probes ranged in size from 0.5 to 3.7, 0.9 to 3.8, and 0.6 to 2.4 kilobase pairs (kbp), respectively.

Dot hybridization. DNA samples from healthy plants and plants infected with various MLOs were prepared as previously described (41). We used nine AY1 and 11 BB MLO-cloned DNA probes in dot hybridization to study the genetic interrelatedness among AY1 MLO, BB MLO, and 23 other MLOs, including many previously identified AY MLOs and MLOs suspected to be related to AY MLO. Two of the 20 DNA probes were derived from MLO extrachromosomal (plasmid) DNA. Dot hybridization and signal detection were performed as described elsewhere (9,35,39). Approximately equal amounts of denatured nucleic acid prepared from AY1, BB, and other MLO-infected plants were blotted onto

TABLE 1. Summary of dot hybridization analysis of mycoplasmalike organism (MLO) strains probed with DNA cloned from MLO strains AY1, BB, and CN1

MLO probe[a]	Number of probes hybridizing with nucleic acid from:														
	AY1	DAY	SAY2	TLAY2	OKAY3	CY2	NYAY	CPh	BB	OKAY1	AY27	CN13	CN1	NJAY	NAY
AY1 (9)	9	9	9	9	9	9	9	9	7	7	8	7	9	9	9
BB[b] (11)	9	9	9	9	9	9	9	11	11	11	11	11	11	11	11
CN1[c] (12)	12	ND	ND[d]	ND	ND	ND	ND	ND	12	ND	ND	ND	12	ND	ND

	MNAY	MBS	BB_I	CP	PWB	AshY	WX	CX	VR	EY	H_V	H_L	H_A	H_C	H_T
AY1 (9)	9	3	9	1	1	2	1	0	0	0	0	0	0	0	0
BB (11)	11	8	11	2	2	2	1	2	0	0	0	0	0	0	0
CN1 (12)	ND	ND	ND	1	1	1	2	2	0	0	0	ND	ND	ND	ND

[a] Total number of probes used is in parentheses.
[b] Numbers are based on data in reference 39, except for strains DAY, SAY2, TLAY2, OKAY3, CY2, NYAY, OKAY1, AY27, NJAY, NAY, MNAY, and for H_L and H_A.
[c] Numbers are based on data in reference 9.
[d] ND, not determined.

nitrocellulose membranes (undiluted nucleic acid, 4 μg per spot), baked, and hybridized with each of the cloned AY1 and BB MLO DNA probes. The presence of a sufficient quantity of detectable MLO-specific DNA in each sample spot was verified separately by hybridization to a homologous DNA probe (data not shown). Hybridizations were performed at 50–52 C in the presence of 45% formamide as described (39). Posthybridization washes of nitrocellulose membranes were done twice (15 min each) at 50 C.

Southern hybridization and RFLP analyses. DNA samples (about 2 μg of total nucleic acid per sample) were doubly digested with *Eco*RI and *Hin*dIII or singly digested with *Eco*RI restriction endonucleases, at 37 C for 4 h to overnight, electrophoresed through a 0.7% agarose gel, alkali denatured, and transferred to a nitrocellulose membrane by the method of Southern as described by Maniatis et al (46). The membranes were air dried, baked, prehybridized, and hybridized with biotin-labeled DNA probes at 50 C.

Twenty-two MLO cloned chromosomal DNA probes (eight AY1 MLO, eight BB MLO, and six CN1 MLO probes) were used for RFLP analyses. For each of the 22 probes, the similarity coefficient (F) of strains x and y was calculated as:

$$F = 2N_{xy}/(N_x + N_y)$$

in which N_x and N_y are the number of fragments in strain x and y, respectively, and N_{xy} is the number of fragments shared between the two strains (51). The F values are the means from the 22 hybridization probes. Cluster analysis was done by the unweighted pair-group method for the F means of all strain comparisons (NTSYS-pc program, Exeter Pub. Ltd., Setauket, NY).

Polymerase chain reactions. We used two oligonucleotide primer pairs previously developed (54,55) for two cloned MLO (AY1) DNA probes, pAY18 and pAY19, in polymerase chain

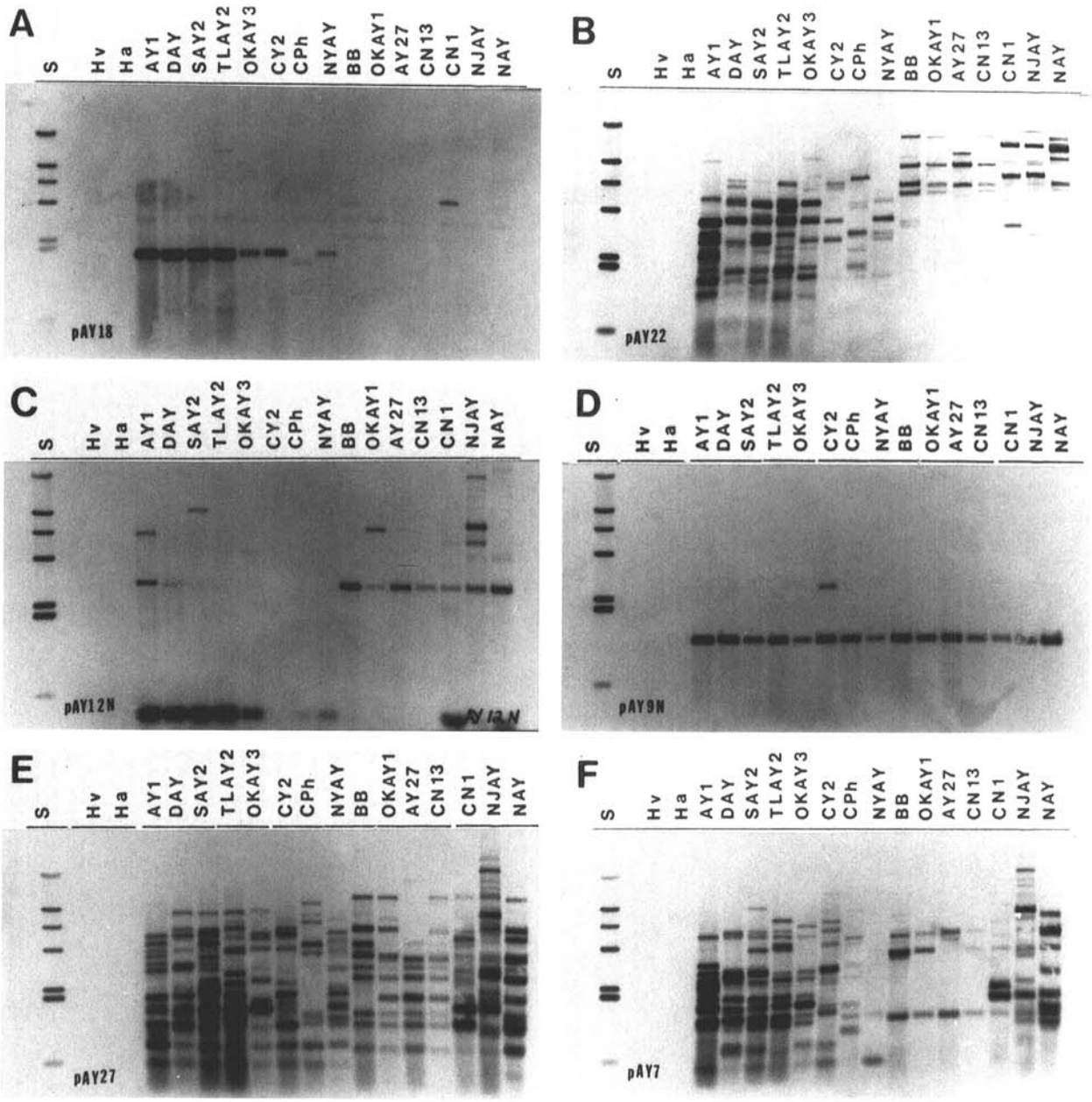

Fig. 1. Southern hybridization analyses of genomic DNAs prepared from healthy plant tissue and plant tissue infected with mycoplasmalike organisms (MLOs). DNA samples were digested with the restriction endonucleases *Eco*RI and *Hin*dIII and hybridized with cloned aster yellows (AY1) MLO DNA probes (pAY18, pAY22, pAY12N, pAY9N, pAY27, and pAY7). Lane S, biotinylated lambda phage DNA *Hin*dIII digest with molecular weights (top to bottom) in kilobase pairs: 23, 9.4, 6.6, 4.4, 2.3, 2.0, and 0.6; H_V is healthy periwinkle DNA; H_A is healthy China aster DNA. MLO strain descriptions are the same as in the text.

reactions (PCR) to further analyze sequence homology among strains in the AY MLO strain cluster. The sequences of the two oligonucleotide primer pairs are as follows: AY18p, 5′ AAC CCA AAC TAT CCA AAG 3′; AY18m, 5′ TGT TTC TAC TTC TTC TTG 3′; AY19p, 5′ TAA CAT CAG AAT AAA TGG 3′; AY19m, 5′ GAC TTA CGT TGG TGA AGG 3′.

For the PCR, total nucleic acid samples extracted from healthy or MLO-infected tissues were diluted in TE buffer (50 mM Tris-HCl, 5 mM EDTA, pH 8) to give the final concentration of 20 ng/μl. The reactions were performed as previously described (54) with Amplitaq DNA polymerase (U.S. Biochemicals, Cleveland, OH) in 50 μl vol of reaction mixture containing 1 μl of the dilute nucleic acid sample, PCR buffer (1× = 10 mM Tris-HCl, pH 8.3, 50 mM KCl, 1.5 mM $MgCl_2$, 0.001% gelatin), 200 μM each dNTP, 0.4 pM each primer pair, and 1.25 units of Amplitaq. Thirty-five PCR cycles were conducted in an automated thermocycler (Perkin-Elmer Cetus, Norwalk, CT). The following parameters were used: 1 min (2 min for first cycle) denaturation step at 94 C, annealing for 2 min at 50 C, and primer extension for 3 min at 72 C. Control tubes without DNA template were included in each experiment as negative controls. After amplification, a 10-μl aliquot from each sample was electrophoresed in a 1.0% agarose gel and visualized by staining with ethidium bromide and UV illumination.

RESULTS

Identification of strains belonging to the AY MLO strain cluster by dot hybridization. Nine AY1 and 11 BB MLO-cloned DNA probes were used in dot hybridization under conditions of high stringency (52 C). Seven to nine of the nine AY1 MLO DNA probes and nine to 11 of the 11 BB MLO probes hybridized, respectively, with nucleic acid prepared from AY1, DAY, SAY2, TLAY2, OKAY3, CY2, NYAY, CPh, BB, OKAY1, AY27, CN13, CN1, NJAY, NAY, MNAY, and BB_1 MLO-infected plants (Table 1). Three of the nine AY1 MLO probes and eight of the 11 BB MLO probes hybridized with nucleic acid from MBS MLO. Two or fewer of the nine AY1 or 11 BB MLO probes hybridized with nucleic acid from CP, PWB, AshY, and CX MLOs. None of the 20 probes hybridized with the nucleic acid from VR or EY MLO. Thus, in addition to the previously identified members (9,39), 11 other MLO strains (i.e., DAY, SAY2, TLAY2, OKAY3, CY2, NYAY, OKAY1, AY27, NJAY, and NAY) were identified as new members of the AY MLO strain cluster.

RFLP analysis. Representative RFLP patterns that resulted from Southern hybridization of digested DNAs from 15 MLO strains in the AY MLO strain cluster by the use of 22 different MLO DNA probes are shown in Figures 1 and 2 (AY1 MLO probes), Figure 3 (BB MLO probes), and Figure 4 (CN1 MLO probes). DNA samples in Figures 1, 3, and 4 were doubly digested with the restriction endonucleases, *Eco*RI and *Hin*dIII. DNA samples in Figure 2 were singly digested with *Eco*RI. Similarity coefficients (F) derived by RFLP analysis ranged from 0.19 to 0.93, indicating genetic heterogeneity of these 15 MLO strains (Table 2). On the basis of similarity coefficients, the 15 MLO strains were broadly classified into three groups. The three groups represented three distinct types of RFLP patterns. Type I (F = 0.71–0.93) consisted of seven MLO strains: BB, OKAY1, AY27, CN13, CN1, NJAY, and NAY MLOs. Type II (F = 0.61–0.89) consisted of seven MLO strains: AY1, DAY, SAY2, TLAY2, OKAY3, CY2, and NYAY MLOs. Type III included one MLO strain, CPh. The CPh MLO was distantly related to type I strains ($F \leq 0.23$) and to type II strains ($F \leq 0.43$). Among strains in type I, CN1, NJAY, and NAY MLOs were moderately related ($F \leq 0.79$) to one another and to the other MLO strains ($F \leq 0.81$), BB, OKAY1, AY27, and CN13. Among strains in type II, six strains (i.e., AY1, DAY, SAY2, TLAY2, OKAY3, and NYAY) were more related (F = 0.75–0.89) to one another than to strain

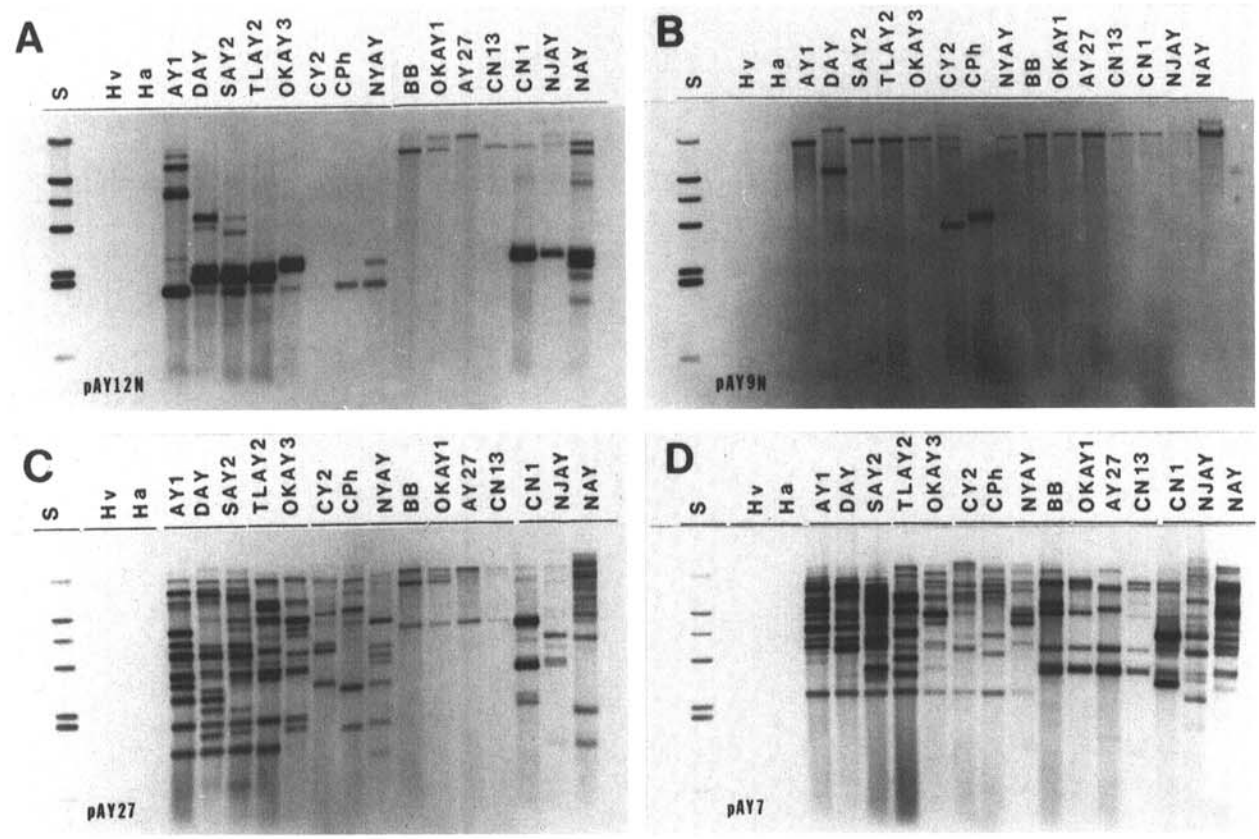

Fig. 2. Southern hybridization analyses of genomic DNAs prepared from healthy plant tissue and plant tissue infected with mycoplasmalike organisms (MLOs). DNA samples were digested with the restriction endonuclease *Eco*RI and hybridized with cloned aster yellows (AY1) MLO DNA probes (pAY12N, pAY9N, pAY27, pAY7). Lane S, biotinylated lambda phage DNA *Hin*dIII digest with molecular weights (top to bottom) in kilobase pairs: 23, 9.4, 6.6, 4.4, 2.3, 2.0, and 0.6; H_V is healthy periwinkle DNA; H_A is healthy China aster DNA. MLO strain descriptions are the same as in the text.

CY2 (F = 0.61–0.72). A best fit dendrogram, with a matrix correlation coefficient of 0.99, is yielded by cluster analysis of the similarity coefficients (Fig. 5). This analysis resulted in three major groups and at least 13 subgroups. The three major groups are equivalent to the three types described above.

RFLP patterns of strains in a given type are similar but not identical (Figs. 1–4). Each strain had its own unique collective pattern. However, there were sequences (or banding patterns) common to MLO strains in a given type (e.g., Figs. 1A,C, 3A,C–E, 4C,D), to strains in two types (e.g., Figs. 1C,E, 4A,B), or to all strains in the AY MLO strain cluster (e.g., Figs. 1D, 3B). For example, in Figure 1D, there was a band (1.4 kbp) common to strains in all three types, whereas in Figure 4A, there was a band (1.3 kbp) common to strains in type I, and there were two bands (3.6 and 0.6 kbp) common to strains in types II and III. Multiple banding patterns were common in all 15 MLO strains examined, particularly when probes pAY22 (Fig. 1B), pAY27 (Fig. 1E), pAY7 (Fig. 1F), pBB50 (Fig. 3D), and pBB111 (Fig. 3F) were used for the assay. The multiple bands of these strains were consistent with different DNA preparations (some were digested for more than 24 h), suggesting the presence of similar or repetitive sequences in multiple regions of the MLO genome, rather than incomplete digestion of the DNA. To further corroborate this phenomenon, we used four probes (pAY12N, pAY9N, pAY27, and pAY7) to hybridize DNA samples that were singly digested with the restriction endonuclease *Eco*RI. The RFLP patterns (Fig. 2) were compared with those (Fig. 1C–F) from hybridizations with DNA samples doubly digested with *Eco*RI and *Hin*dIII. A sequence or DNA band (about 0.5 kbp) as shown in Figure 1C (AY1, DAY, SAY2, TLAY2, OKAY3, NYAY, CN1, and NAY) and a sequence (about 1.4 kbp) as shown in Figure 1D (DAY, CY2, and NAY) were actually present in more than one copy in the genomes of these MLO strains (Fig. 2A,B). Multiple banding patterns of many of the 15 MLO strains were evident when DNA samples were probed with pAY27 or pAY7, although the number of bands of BB, OKAY1, AY27, and CN13 MLOs were reduced when DNA samples that were singly digested with *Eco*RI (Fig. 2C vs. Fig. 1E) were probed

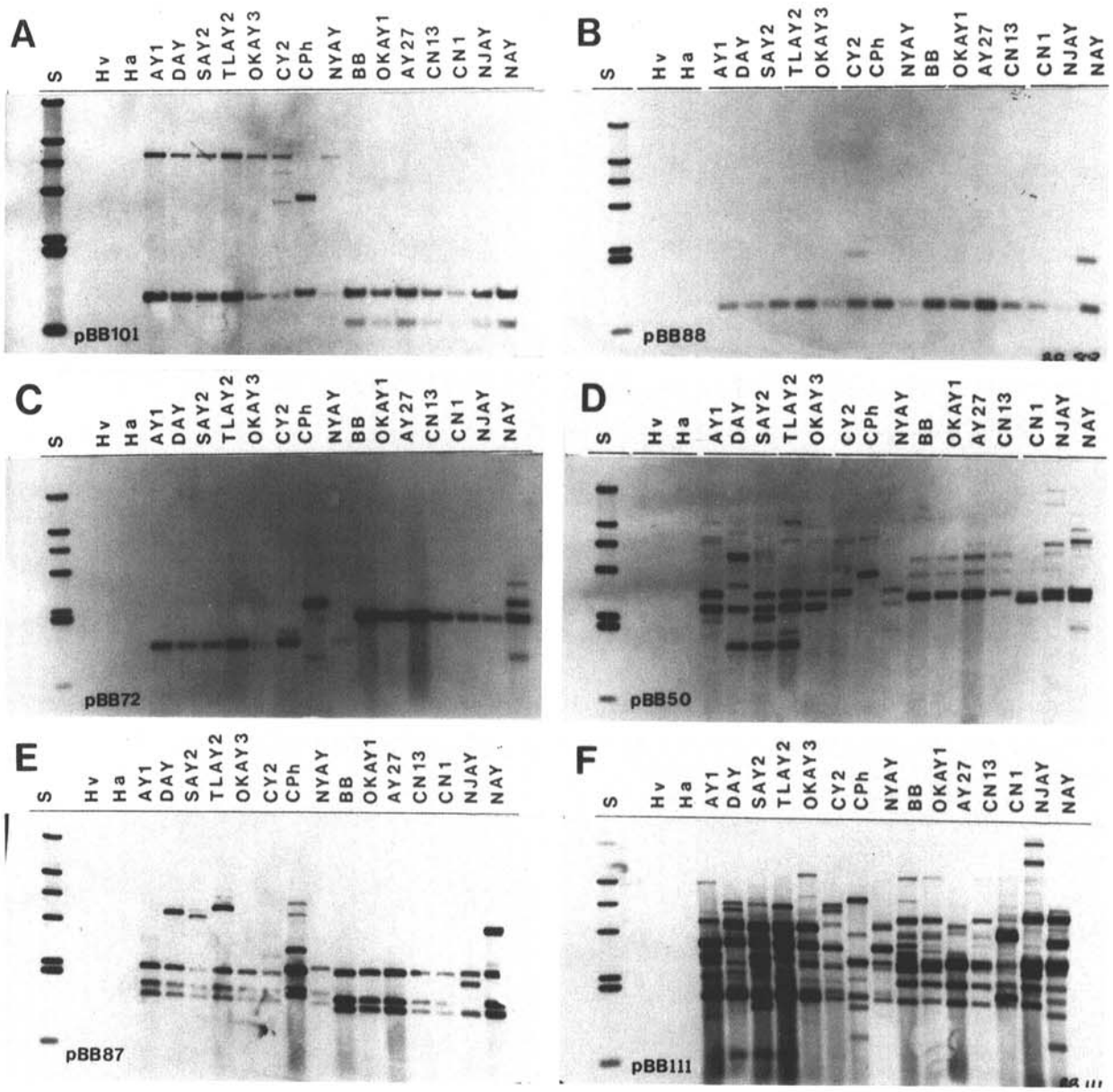

Fig. 3. Southern hybridization analyses of genomic DNAs prepared from healthy plant tissue and plant tissue infected with mycoplasmalike organisms (MLOs). DNA samples were digested with the restriction endonucleases *Eco*RI and *Hin*dIII and hybridized with cloned tomato big bud (BB) MLO DNA probes (pBB101, pBB88, pBB72, pBB50, pBB87, pBB111). Lane S, biotinylated lambda phage DNA *Hin*dIII digest with molecular weights (top to bottom) in kilobase pairs: 23, 9.4, 6.6, 4.4, 2.3, 2.0, and 0.6; H_V is healthy periwinkle DNA; H_A is healthy China aster DNA. MLO strain descriptions are the same as in the text.

with pAY27.

Polymerase chain reaction. With the primer pair, AY18p and AY18m, all type II strains (AY1, DAY, SAY2, TLAY2, OKAY3, CY2, NYAY) and the type III strain CPh each produced a major band corresponding to 1.8 kbp, whereas type I strains (BB, OKAY1, AY27, CN13, CN1, NJAY) each produced a major band corresponding to 2.5 kbp. With the primer pair, AY19p and AY19m, all type II strains and two type I strains (CN1 and NJAY) produced a 1.2-kbp fragment, which was not produced in reactions with the remaining strains. No specific PCR products were obtained in any of the reactions with the other MLO strains, CX, WX, EY, VR, AshY, CP, and PWB (data not shown) and in the control reactions with nucleic acid from healthy plants (Fig. 6) or with water without template DNA (data not shown). The lack of PCR product from the NAY DNA preparation is probably due to the presence of inhibitors in the aster extract. In a separate experiment (data not shown) with NAY DNA extracted from clover, a 1.2-kb fragment was produced with primer pair, AY19p and AY19m, and no product was produced with primer pair, AY18p and AY18m.

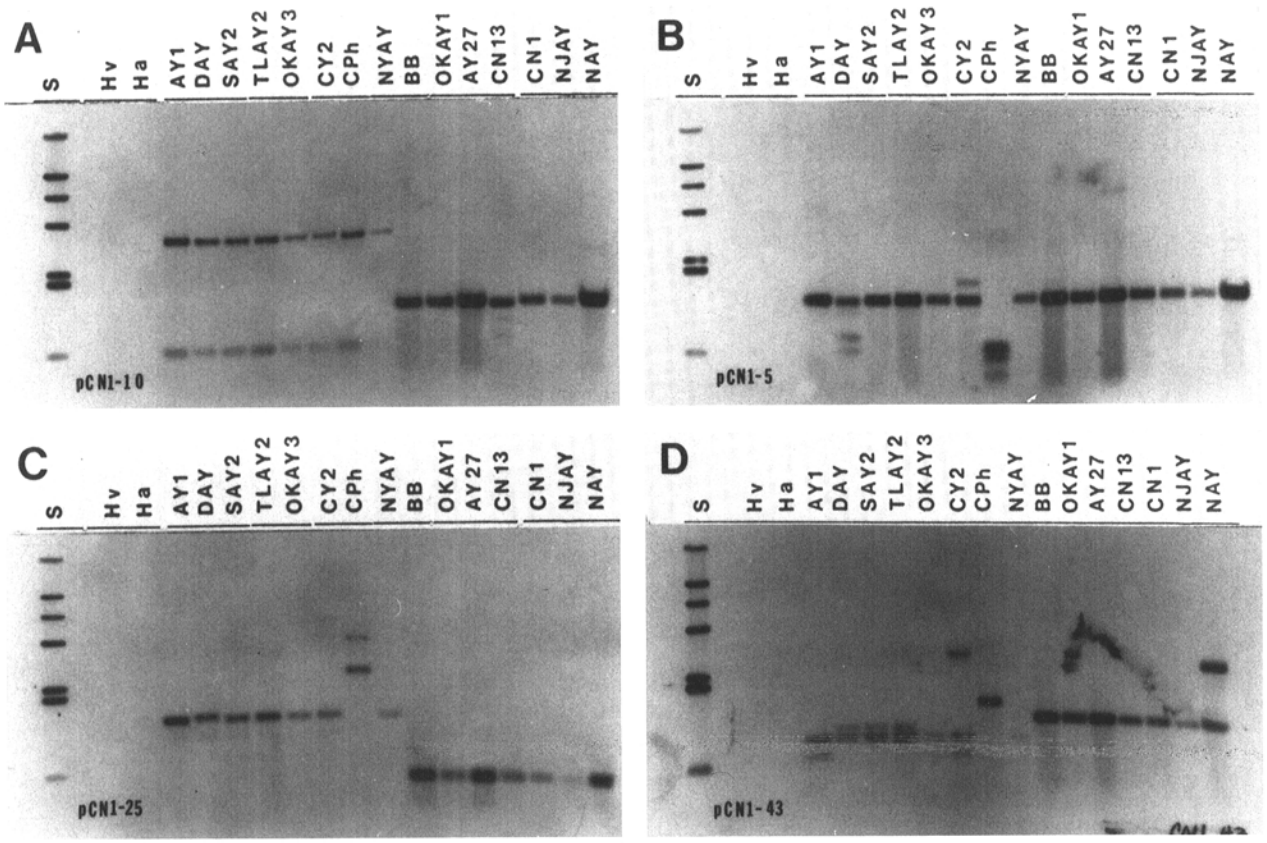

Fig. 4. Southern hybridization analyses of genomic DNAs prepared from healthy plant tissue and plant tissue infected with mycoplasmalike organisms (MLOs). DNA samples were digested with the restriction endonucleases *Eco*RI and *Hin*dIII and hybridized with cloned periwinkle little leaf (CN1) MLO DNA probes (pCN1-10, pCN1-5, pCN1-25, pCN1-43). Lane S, biotinylated lambda phage DNA *Hin*dIII digest with molecular weights (top to bottom) in kilobase pairs: 23, 9.4, 6.6, 4.4, 2.3, 2.0, and 0.6; H_V is healthy periwinkle DNA; H_A is healthy China aster DNA. MLO strain descriptions are the same as in the text.

TABLE 2. Similarity coefficients, F[a], derived from restriction fragment length polymorphism analysis of strains in the aster yellows mycoplasmalike organism (AY MLO) cluster

	Selected strains of AY MLO cluster														
Strain	AY1	DAY	SAY2	TLAY2	OKAY3	CY2	NYAY	BB	OKAY1	AY27	CN13	CN1	NJAY	NAY	CPh
AY1	1.00														
DAY	0.75	1.00													
SAY2	0.83	0.84	1.00												
TLAY2	0.77	0.80	0.84	1.00											
OKAY3	0.87	0.77	0.81	0.80	1.00										
CY2	0.70	0.72	0.66	0.61	0.62	1.00									
NYAY	0.89	0.79	0.83	0.80	0.84	0.67	1.00								
BB	0.30	0.26	0.25	0.28	0.26	0.20	0.25	1.00							
OKAY1	0.30	0.25	0.25	0.28	0.26	0.20	0.25	0.92	1.00						
AY27	0.30	0.29	0.27	0.28	0.26	0.23	0.26	0.88	0.89	1.00					
CN13	0.29	0.28	0.26	0.28	0.26	0.20	0.25	0.93	0.93	0.89	1.00				
CN1	0.33	0.30	0.26	0.31	0.30	0.21	0.30	0.79	0.81	0.79	0.81	1.00			
NJAY	0.30	0.29	0.30	0.30	0.31	0.23	0.28	0.74	0.76	0.71	0.75	0.79	1.00		
NAY	0.28	0.27	0.34	0.26	0.28	0.25	0.26	0.71	0.74	0.71	0.74	0.76	0.68	1.00	
CPh	0.39	0.42	0.39	0.43	0.39	0.36	0.39	0.21	0.19	0.23	0.21	0.21	0.21	0.21	1.00

[a]Each coefficient, F, is the average of 22 observations (analysis with 8, 8, and 6 probes derived from tomato big bud (BB), Maryland aster yellows (AY1), and periwinkle little leaf (CN1) MLOs, respectively, and the restriction endonucleases *Eco*RI and *Hin*dIII).

DISCUSSION

MLOs are transmitted by insect vectors and are believed to be the cause of disease in several hundred plant species (49). Because MLOs cannot be cultured in vitro, diagnosis of MLO-associated diseases and identification of MLOs in the past were primarily based on a positive microscopic detection of MLOs in diseased phloem tissue (1,16,22,25) and on their biological characteristics, which included MLO-insect vector relationships, plant host range, and the characteristic symptoms they induce in host plants (4–7,20,31,32,44,47,48,52,53,57,59). Classification systems for MLO-associated diseases or MLOs were recently proposed on the basis of symptomatology in infected plants (6,26,48). For example, Chiykowski and Sinha (6) classified MLOs into two major categories on the basis of floral symptoms. Category 1 included those MLOs causing phyllody and/or virescence; category 2 included those causing reduction in flower size and color but never phyllody or virescence. MLOs in each category were then differentiated on the basis of specificity of insect transmission. Reliance on biological properties to identify and classify MLOs, however, is time-consuming and not completely applicable to all MLOs. For example, insect vectors of many MLOs are still unknown.

The classification system for MLOs presented in this study involved two steps of genomic DNA analysis. Dot hybridization analysis was conducted for a preliminary separation or identification of major MLO strain clusters, as done previously (9–12,35,39,41). RFLP analysis was employed for subsequent differentiation of subclusters (types) and strains in a given strain cluster. On the basis of the results from dot hybridizations with DNA probes derived from AY1, BB, or CN1 MLO, 15 MLO strains among others were identified and classified as members of the AY MLO strain cluster (9,35,39). The 15 MLO strains examined in this study included 10 MLO strains that had each previously been designated either an eastern or a western AY or an undesignated AY strain, and five strains were associated with other diseases (1,4,7,9). Nearly all of the 22 probes used in this study hybridized with DNA from each of the 15 AY strains. In contrast, the CP, PWB, AshY, WX, CX, VR, and EY MLO DNAs hybridized with few of these probes. The results are consistent with previous work (9,35,39), indicating that AY1, BB, CN1, CPh, and CN13 MLOs belong to the AY MLO strain cluster. MLO strains in the AY MLO strain cluster are closely related to one another and are distantly related to other MLOs. Analyses of RFLP patterns with 22 different chromosomal DNA probes derived from three members (AY1, BB, and CN1) in the AY MLO strain cluster revealed that the 15 strains could be grouped into three discrete groups, which we call types. There were homologous sequences commonly present in all three strain types (Figs. 1D, 3B) or in any two of the three strain types (Figs. 1C, 2D, 3A, 4A,B). These sequences that are conserved among strains in all three types, or any two types, evidently are not shared by any other MLO strains associated with other yellows diseases and are unique to the AY MLO strain cluster. Thus, selected DNA probes (e.g., pAY9N, pBB88, pBB101, and pCN1-25) can be employed in RFLP analyses for identification of all three types (using pAY9N, pBB88) in the AY MLO strain cluster and for differentiation among the types (using pBB101, pCN1-25) within this cluster.

Genotypic diversity in the AY MLO strain cluster is evident, because none of the 15 strains exhibited the same or nearly identical RFLP patterns. The closest strain pair (e.g., BB and CN13) had a similarity coefficient of 0.93. The diverse RFLP patterns could not be due to a mixture of strains that represent two or more types of MLOs present in some DNA samples employed in this study, because not a single RFLP fingerprint exhibited patterns characteristic of more than one type. PCR analyses with specific primers also indicated that the MLO populations present in each strain are essentially homogeneous; none of the PCR products for either type I or II strains contained both DNA fragments (e.g., 1.8 and 2.5 kbp; Fig. 6A), which would indicate a mixed population. Genotypic diversity in the AY MLO strain cluster may result from genetic exchanges among MLOs that share common vectors and plant hosts. Although geographical or biological isolation (association with their

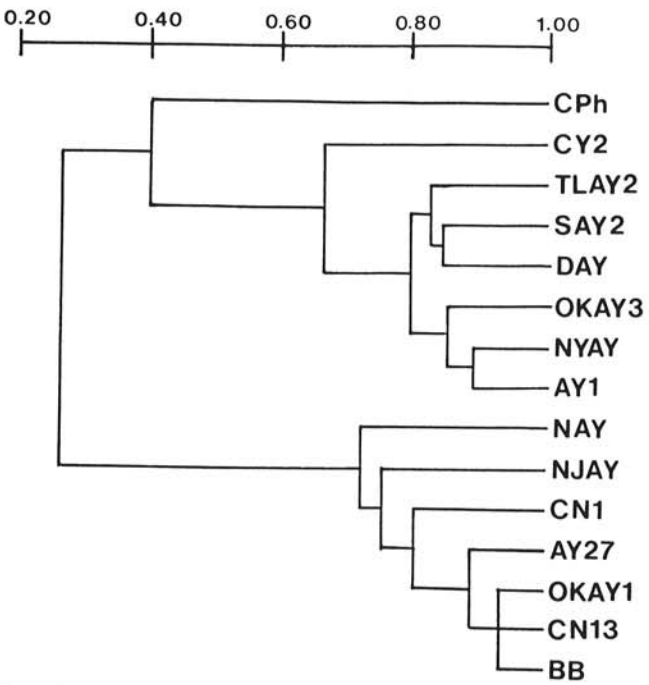

Fig. 5. Dendrogram obtained by cluster analysis of similarity coefficients, *F*, derived from restriction fragment length polymorphism analysis of strains in the aster yellows (AY) mycoplasmalike organism (MLO) strain cluster. Scale refers to the similarity index. MLO strain descriptions are the same as in the text.

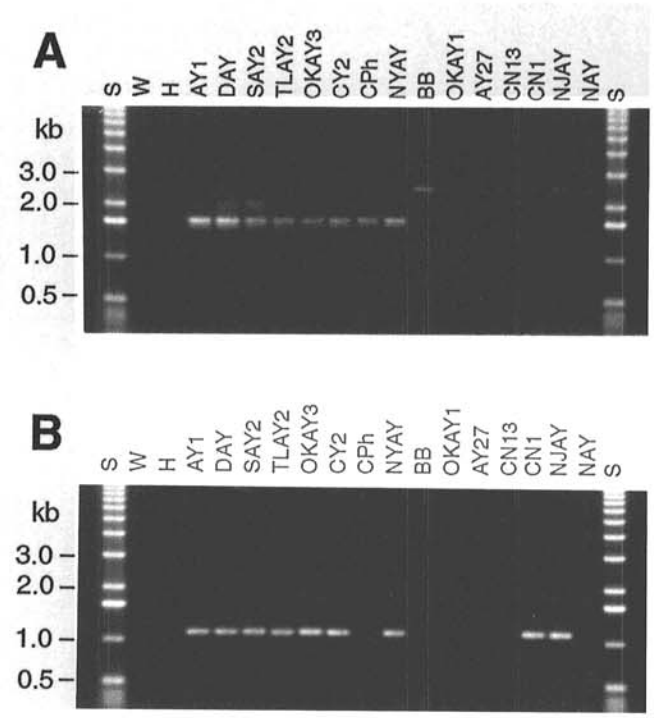

Fig. 6. Polymerase chain reactions (PCR) analysis of DNA extracted from healthy plant tissue and plant tissue infected with mycoplasmalike organisms (MLOs). Reaction products are the result of PCR amplification with primer pairs derived from **A,** pAY18 and **B,** pAY19. Figure shows results from electrophoresis of reaction products in a 1.2% agarose gel after staining with ethidium bromide. Lane S, DNA ladder; W, water control; H, healthy *Catharanthus roseus*; AY1–NAY are PCR products from MLO-infected plants (strain descriptions are the same as in the text).

TABLE 3. Strains in the aster yellows mycoplasmalike organism (AY MLO) strain cluster: host origin, geographical location, and types of symptoms induced in periwinkle host

MLO strain	Strain type	Original disease name[a]	Symptoms induced[b]	Origin		Reference
				Host(s)	Location	
BB	I	Tomato big bud	Sf, Pr	Tomato	Arkansas	7
OKAY1	I	Western AY	V, Ph, Pr, WB	Lettuce	Oklahoma	17
AY27	I	Western AY	V, Ph, WB	Aster	Canada	14
CN13	I	Periwinkle little leaf	V, Ph, Pr, WB	Periwinkle	Connecticut	9
CN1	I	Periwinkle little leaf	V, Ph, Pr, WB	Periwinkle	Connecticut	9
NJAY	I	New Jersey AY	V, Ph, WB	Lettuce	New Jersey	45
NAY	I	Eastern AY	V, Ph, WB	Lettuce	Canada	5
AY1	II	Maryland AY	V, Ph, WB	Periwinkle	Maryland	35
DAY	II	Western dwarf AY	V, Ph	Clover	California	19
SAY2	II	Western severe AY	V, Ph, WB	Celery, lettuce	California	19
TLAY2	II	W. Tulelake AY	Sf	Potato	California	19
OKAY3	II	Western AY	Sf	Carrot	Oklahoma	17
CY2	II	Chrysanthemum Y	V, Ph	Chrysanthemum	Italy	1
NYAY	II	Eastern AY	V, Ph, WB	Lettuce	New York	C. Hiruki
CPh	III	Clover phyllody	V, Ph	Clover	Canada	4

[a] Y = yellows.
[b] Sf = small flower, Pr = proliferation, V = virescence, Ph = phyllody, WB = witches'-broom.

preferential insect vectors) tends to preserve the strain characteristics, the overlapping plant host ranges and dual or multiple vectorships provide vast opportunities for MLO strains to exchange their genetic information. Also, genetic diversity in the AY MLO strain cluster may be attributable to genomic rearrangement. RFLP analysis indicated that regions of repetitive sequence homology are evident in the genomes of all three strain types. For instance, probes pAY27 and pAY9 hybridized repetitively to multiple regions of the genomic DNA in all three strain types. The presence of repetitive sequence homology in all three strain types may indicate frequent rearrangement in composition of the genomic DNAs of members in the AY MLO strain cluster.

In this work, we have reclassified several previously designated AY strains and identified several MLO strains associated with other yellows diseases as members of the AY MLO strain cluster. For example, type I AY strains include two strains (NAY and NJAY) previously designated eastern AY or New Jersey AY (5,45), two strains (AY27 and OKAY1) previously designated western AY (14,17), and three strains (BB, CN1, and CN13) associated with other diseases (7,9) (Table 3). This classification is in good agreement with results obtained by monoclonal antibody typing (42) or PCR analysis (this study; 54). Both monoclonal antibody assays and PCR analyses distinguished type I MLO strains from type II and III strains (PCR analyses see Fig. 6). Results from analyses of 16S rRNA sequences reaffirm the close phylogenetic relationship among members in the AY MLO strain cluster and their divergence from other MLOs (I.-M. Lee and R. E. Davis, *unpublished data*). However, our new classification is not completely consistent with the previous classification systems that are based solely on the biological properties (e.g., host range and/or symptomatology).

Extensive symptom variations induced in host plants (*C. roseus*) (Table 3) by strains in the AY MLO strain cluster, or within a single type, strongly suggest that classification of strains by symptomatology does not always coincide with phylogenetic relationships. It is conceivable that symptom expression in host plants infected by MLOs may be controlled by variable genes that represent only a small portion of the entire genome. It is not uncommon for two distantly related MLOs (e.g., VR [20] and DAY [57]) to cause remarkably similar symptoms in some common hosts, whereas two closely related MLOs (e.g., SAY and DAY) can induce very different symptom types (I.-M. Lee and R. E. Davis, *unpublished*). Kunkel (30) described that abnormal production of secondary shoots, upright growth, stunting, and virescent flowers were typical symptoms induced by the eastern strain of AY MLO. We have observed that some strains that are closely related to the eastern AY MLO (based on RFLP analysis in this study) may lack one or more of those characteristic symptom types. For example, BB MLO (a type I member of the AY MLO strain cluster) does not induce virescent flowers. In view of the phenotypic variability of MLO strains in the AY MLO strain cluster, it is conceivable that more MLO strains that cause symptoms atypical of traditional AY will be identified and included in the AY MLO strain cluster, whereas some MLOs previously identified as AY MLOs on the basis of symptomatology will be found not to be AY MLOs. Further analysis of suspect AY MLOs from all over the world would certainly expand the scheme.

The recent development of MLO-specific serological and DNA hybridization assays has advanced diagnostic technologies for MLOs; it has provided a rapid and reliable means for detection and identification of MLOs (2,3,8–12,14,15,23,24,26–29,33–43, 45,50,58,60). The use of cloned MLO DNAs as probes in DNA hybridization assays and in RFLP analyses permits investigation of genetic relatedness among various MLO strains and differentiation among MLOs. This approach has resulted in significant information about the genetic interrelatedness among those MLOs and recognition of the AY MLO and several other distinct MLO strain clusters (35,38,40,41,43). These studies have provided the framework for a genotype-based scheme for identification and classification of MLOs.

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