Universal Amplification and Analysis of Pathogen 16S rDNA for Classification and Identification of Mycoplasmalike Organisms

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ABSTRACT

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Regions representing about 80% of the 16S rDNA sequences of 40 mycoplasmalike organism (MLO) strains from North America, Asia, and Europe were amplified by polymerase chain reaction (PCR) using a primer pair designed on the basis of an MLO 16S rRNA gene. This primer pair detected every MLO examined from infected periwinkle (*Catharanthus roseus*) and some other plants. No PCR products were obtained in samples containing DNA extracted from healthy plants. The partial 16S rDNA sequences amplified from these various MLOs were compared through restriction fragment length polymorphism (RFLP) analyses. Based on similarity coefficients derived from RFLP analyses, these 40 MLOs could be classified into nine distinct 16S ribosomal RNA (16Sr) groups and 14 subgroups, including five groups that coincide with MLO strain clusters previously delineated on the basis of dot hybridization analysis using randomly cloned chromosomal DNA probes. Type MLO strains designated for each group and subgroup were as follows: 16SrI-A, tomato big bud; 16SrI-B, Maryland aster yellows; 16SrI-C, clover phyllody; 16SrI-D, paulownia witches'-broom; 16SrI-E, blueberry stunt; 16SrII, peanut witches'-broom; 16SrIII-A, Canada peach X; 16SrIII-B, clover yellow edge; 16SrIV, palm lethal yellowing; 16SrV, elm yellows; 16SrVI, clover proliferation; 16SrVII, ash yellows; 16SrVIII, loofah witches'-broom; and 16SrIX, pigeon pea witches'-broom.

Additional keywords: MLO strain cluster, Mollicutes, phylogenetic relationship.

Mycoplasmalike organisms (MLOs) are associated with diseases in several hundred plant species (20). Thus far, none has been cultured in vitro (13). Historically, disease or pathogen identification and classification have primarily relied on comparative studies of their biological properties (3). Such procedures are time-consuming and often subjective and unreliable. In the past 7–8 yr, the development of MLO-specific molecular probes for serological and DNA hybridization assays has provided rapid and reliable means for MLO diagnosis and classification (2,13). DNA hybridization analyses using cloned DNA probes derived from various MLOs have made it possible to study the genetic interrelatedness among these MLOs, leading to the recognition of several distinct MLO strain clusters (5–7,12–16). Each strain cluster consists of MLO strains that share extensive DNA sequence homology with one another and are distinct from strains in the other MLO strain clusters. Strain (or genotype) differentiation among members in a given MLO strain cluster has been achieved through restriction fragment length polymorphism (RFLP) analysis of each MLO chromosomal DNA by using selected DNA probes (14–16). These results have formed the framework of a classification scheme for identification and differentiation of the uncultured MLOs. However, thus far DNA fragments have been cloned from only a limited number of MLOs. The unavailability of cloned DNA fragments from many uncharacterized MLOs and the difficulty in obtaining desired concentrations of MLO DNA from some infected host plants can pose major problems in this type of assay.

Recently, work from our laboratory and others has demonstrated that sensitive detection and differentiation of MLO strains can be achieved by polymerase chain reaction (PCR) assay (1,6,8–10,22). Specific oligonucleotide primer pairs designed on the basis of well-characterized cloned MLO DNA sequences or from 16S rRNA sequences of animal mycoplasmas and MLOs have been employed in PCR for detection and/or identification of MLOs from infected plant tissues (1,9). The use of PCR for amplification of 16S rDNA sequences has advanced our ability to detect a much wider array of MLO strains from low-titer hosts. Recently, Deng and Hiruki reported a primer pair that could be used in PCR to detect 16S rDNA of several MLOs in infected periwinkle (9). Ahrens and Seemüller reported a primer pair that amplified 16S rDNA of a wider array of MLOs from infected plant tissues (1). However, the latter primer pair designed for broad MLO detection was not specific for MLOs. Often, PCR products similar in size were amplified from samples prepared from healthy as well as from infected plant tissues (1). RFLP analyses of the PCR products (16S rDNA fragments) were necessary to distinguish the 16S rDNA fragments of plant host tissue origin from those of MLOs.

The objective of this study was to develop a PCR-based procedure for classification and identification of MLOs. A "universal" primer pair was designed for specific amplification of 16S rDNA sequences from a broad array of MLOs from infected plant tissues, and RFLP analysis of the amplified 16S rDNA was used as the basis for differentiation and classification of these uncultured MLOs. Preliminary reports on these studies were presented earlier (17,18).

MATERIALS AND METHODS

Source of MLOs. Forty MLO strains from North America, Asia, and Europe were used in this study. Maryland aster yellows (AY1) was originally collected from naturally diseased periwinkle (*Catharanthus roseus* (L.) G. Don) at Beltsville, Maryland; and periwinkle yellows (strains SL1, SL5, SL7, and SL8) were originally collected from fields of periwinkle in St. Louis, Missouri. Other MLO strains were provided by the following researchers, who provided each strain separately in periwinkle, in other hosts as indicated, or as DNA samples from plant hosts as indicated: clover proliferation (CP), potato witches'-broom

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(PWB), a strain of Alberta aster yellows (AY27), and a strain of New York aster yellows (NYAY) (C. Hiruki, University of Alberta, Edmonton, Alberta, Canada); strains of Oklahoma aster yellows MLO (OKAY1, OKAY3) (J. Fletcher, Oklahoma State University, Stillwater); a New Jersey strain of aster yellows (NJAY) (T.-A. Chen, Rutgers University, New Brunswick); an eastern strain of aster yellows (NAY) in clover, clover phyllody (CPh), clover yellow edge (CYE) in clover, and Canada peach X-disease (CX) (L. N. Chiykowski, Agriculture Canada, Ottawa, Ontario, Canada); severe aster yellows (SAY2), dwarf aster yellows (DAY), and Tulelake aster yellows (TLAY2) (A. H. Purcell, University of California, Berkeley); tomato big bud (BB) (J. Dale, University of Arkansas, Fayetteville); periwinkle little leaf (CN1 = Orchard-1, CN13 = Orchard-13) (S. M. Douglas, Connecticut Agricultural Experiment Station, New Haven); ash yellows (AshY) and elm yellows (EY1, EY2 [in Chinese elm]) (W. A. Sinclair, Cornell University, Ithaca, New York); 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), Italy elm yellows (EYIta), and hydrangea phyllody (HyPh1) (A. Bertaccini, Instituto di Patologia Vegetale, Bologna, Italy); palm lethal yellowing (LY3) (DNA sample from diseased coconut), pigeon pea witches'-broom (PPWB) (N. A. Harrison, University of Florida, IFAS, REC, Ft. Lauderdale); peanut witches'-broom (PnWB), loofah (*Luffa* spp.) witches'-broom (LfWB), red bird cactus (*Pedilanthus tithymaloides* (L.) Poit) witches'-broom (RBCWB), sweet potato witches'-broom (SPWB), *Ipomoea obscura* witches'-broom (IObWB), and an undesignated MLO (UD1), paulownia witches'-broom (PaWB) (H. J. Su and M. C. Tsai, National Taiwan University, Taipei); a strain of Michigan aster yellows (MIAY 86-7) that infects *Oenothera* spp. (19) (B. B. Sears, Michigan State University, East Lansing); and blueberry stunt (BBS1) (D. C. Ramsdell, Michigan State University, East Lansing).

Primer pairs and PCR conditions. Four oligonucleotide primers (R16F0, R16F2, R16R0, and R16R2) were designed on the basis of 16S rRNA sequence from a strain of Michigan aster yellows MLO (MIAY 86-7), as presented in Figure 1 of reference 19. The oligonucleotide sequences of the four primers and base locations are R16F0, 5′-CTG GCT CAG GAT TAA CGC TGG CGG C-3′ (base 18-42); R16F2, 5′-ACG ACT GCT GCT AAG ACT GG-3′ (base 152-168); R16R0, 5′-GGA TAC CTT GTT ACG ACT TAA CCC C-3′ (base 1479-1503); and R16R2, 5′-TGA CGG GCG GTG TGT ACA AAC CCC G-3′ (base 1373-1397)

Three sets of primer pairs, R16F0R0, R16F0R2, and R16F2R2, were evaluated for MLO detection by PCR. Total nucleic acid samples extracted from healthy or MLO-infected plant tissue as described elsewhere (15) were diluted in sterile deionized water to a final concentration of 20 ng/μl. Each reaction was performed as previously described, using 200 μM deoxynucleotide triphosphates and 0.4–1.0 μM primer pair (22). Thirty-five PCR cycles were conducted in an automated thermocycler (Perkin-Elmer Cetus, Norwalk, CT). The following parameters were used: a 1-min (2-min for the first cycle) denaturation step at 94 C, annealing for 2 min at 50 C, and primer extension for 3 min (10 min in the final cycle) at 72 C. Control tubes with nucleic acid samples from healthy plants or without DNA template were included in each experiment as negative controls. PCR products were analyzed by electrophoresis through a 1% agarose gel followed by staining in ethidium bromide and visualization of DNA bands using a UV transilluminator.

RFLP analyses of PCR products (partial 16S rDNA sequences). 16S rDNA sequences of the 40 MLO strains amplified by PCR using the primer pair R16F2R2 were analyzed by restriction endonuclease digestion. Between 3 and 5 μl (100–200 ng of DNA) of each PCR product was digested separately with the following restriction enzymes according to the instructions of the manufacturer: *Alu*I, *Dra*I, *Eco*RI, *Eco*RII, *Hae*III, *Hha*I, *Hin*fI, *Hpa*I, *Hpa*II, *Kpn*I, *Rsa*I, *Taq*I, and *Tha*I (GIBCO BRL, Gaithersburg, MD), and *Mse*I and *Sau*3A (New England Biolabs, Beverly, MA). The restriction products were then separated by electrophoresis through a 5% polyacrylamide gel and stained in ethidium bromide. DNA bands were then visualized using a UV transilluminator. The RFLP patterns (sum of results analyzed by 15 restriction enzymes) of the 40 MLO strains were compared and analyzed as previously described by Nei and Li (21). Fragments smaller than 30 bp were not included in these analyses. These small fragments were thought to be contributed predominantly by excess primer or primer dimers generated through PCR. 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 (resulting from digestions by 15 enzymes) in strains x and y, respectively, and N_{xy} is the number of fragments shared by the two strains. Cluster analysis was done by the unweighted pair-group method for F means of all strain comparisons (NTSYS-pc program, Exeter Publishing, Ltd., Setauket, NY).

RESULTS

Primer pair for amplification of MLO-specific 16S rDNA sequences. Of the three primer pairs designed for PCR, two primer pairs, R16F0R0 and R16F0R2, amplified 16S rDNA sequences from DNA samples extracted from both healthy and MLO-infected plant tissues (data not shown). The primer pair R16F2R2 amplified a 16S rDNA fragment (about 1.2 kb) from the MIAY 86-7 MLO DNA sample and each DNA sample prepared from all other MLO infected tissues but not from any DNA sample extracted from healthy tissues (Fig. 1).

RFLP analyses of 16S rDNA sequences of MLOs. MLO 16S rDNA sequences were analyzed by separate digestion with 15 different restriction enzymes. RFLP analyses of the 40 MLO 16S rDNA sequences delineated distinct pattern types. Representative RFLP patterns of the 40 MLO 16S rDNA sequences are shown in Figures 2 and 3 and in Table 1. The RFLP pattern types

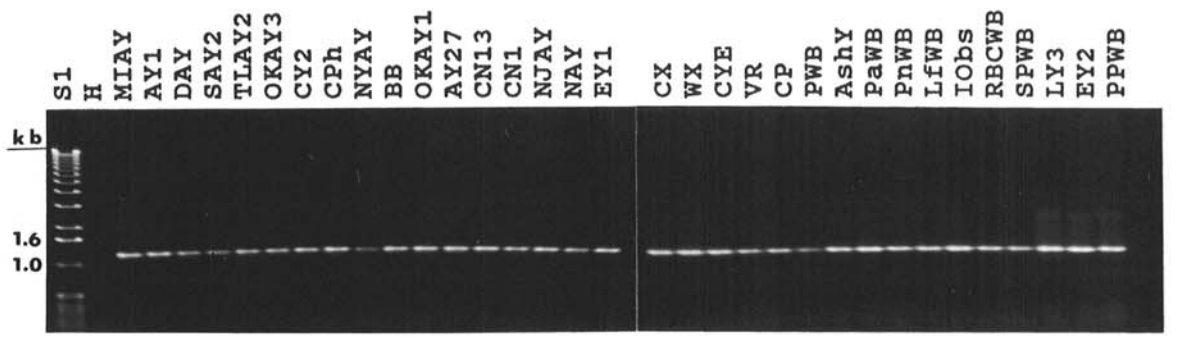

Fig. 1. Polymerase chain reaction (PCR) amplification of a 16S rDNA sequence from various mycoplasmalike organisms using primer pair R16F2R2. PCR products (35 cycles) were separated by electrophoresis through a 1% agarose gel. Lane S1, 1-kb DNA ladder (GIBCO BRL); lane H, healthy *Catharanthus roseus*. All other abbreviations are defined in the text.

delineated by use of the 15 enzymes are summarized in Table 1. Similar RFLP patterns were delineated when restriction enzymes *Eco*RI (Table 1), *Hpa*I (Fig. 3C and Table 1), *Dra*I (Fig. 3L and Table 1), and *Tha*I (Table 1) were used for analysis of the amplified MLO sequences, indicating the presence of conserved restriction sites of these enzymes among the 40 MLOs. More variable RFLP patterns were delineated among these MLOs when restriction enzymes *Mse*I (Fig. 2A, Fig. 3A, and Table 1), *Alu*I (Fig. 2B, Fig. 3B, and Table 1), *Rsa*I (Fig. 3F and Table 1), and *Hha*I (Fig. 2D, Fig. 3I, and Table 1) were used for analysis. The RFLP pattern of the CPh MLO delineated by use of restriction enzyme *Alu*I was unique (Fig. 2B and Fig. 3B). In addition to the five major fragments common to every member of the AY MLO strain cluster, a unique fragment was generated. The combined size of these six fragments was larger than the expected 1.2 kb, indicating the presence of two 16S ribosomal genes with minor differences in the CPh MLO. The possibility of a mixed infection by two MLOs is doubtful, because only one RFLP pattern was observed when many other restriction enzymes were used to analyze the PCR product. In addition, other evidence based on RFLP and PCR analyses using specific probes indicating a single infection was presented elsewhere (14). The presence of two ribosomal genes has been proposed in the SAY2 MLO (11).

Collective RFLP patterns (based on analyses with the 15 restriction enzymes) among the strain MIAY 86-7 and the 15 MLO strains (BB, OKAY1, AY27, CN13, CN1, NJAY, NAY, AY1, DAY, SAY2, TLAY2, OKAY3, CY2, NYAY, and CPh), previously designated as members of the AY MLO strain cluster (14), were identical or very similar (Figs. 2 and 3). Identical or very similar RFLP patterns (Fig. 2) were also observed among MLO strains CX, WX, and CYE (previously designated as members of the peach X MLO strain cluster [16]); between strains CP and PWB (previously designated as members of the CP MLO strain cluster [15]); and among strains EY1, EY2, and EYIta (data not shown) (previously designated as members of the EY MLO strain cluster [13]). The collective RFLP patterns among those designated strain clusters including the AshY MLO strain cluster (7) were distinct from one another. The strains LY3, PPWB, LfWB and the three strains UD1, PnWB, and RBCWB (data not shown) represented another four distinct RFLP pattern groups that were different from those shown by members of the five

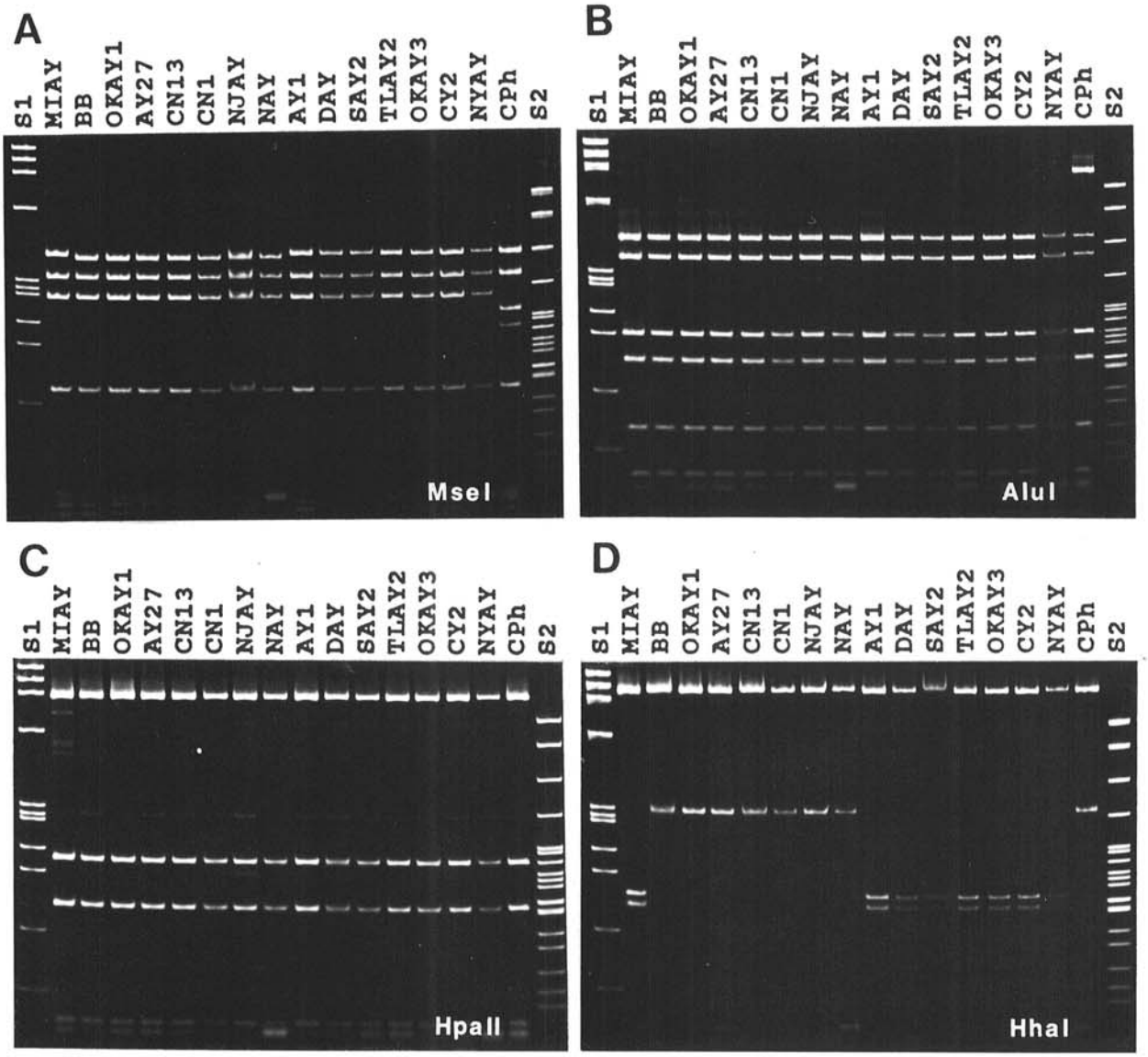

Fig. 2. Restriction fragment length polymorphism analyses of 16S rDNA amplified by polymerase chain reaction using the primer pair R16F2R2 from MIAY and 15 mycoplasmalike organisms (MLOs) previously designated as members of the aster yellows MLO strain cluster. DNA products were digested with restriction enzymes **A,** *Mse*I, **B,** *Alu*I, **C,** *Hpa*II, and **D,** *Hha*I and separated by electrophoresis through a 5% polyacrylamide gel. Lane S1, ϕX174 RF I DNA *Hae*III digest, fragment sizes in base pairs from top to bottom: 1353, 1078, 872, 603, 310, 281, 271, 234, 194, 118, and 72; lane S2, pBR322 DNA *Msp*I digest, fragment sizes in base pairs from top to bottom: 622, 527, 404, 307, 242, 238, 217, 201, 190, 180, 160, 147, 123, 110, 90, 76, 67, 34, 26, 15, 9. All other abbreviations are defined in the text.

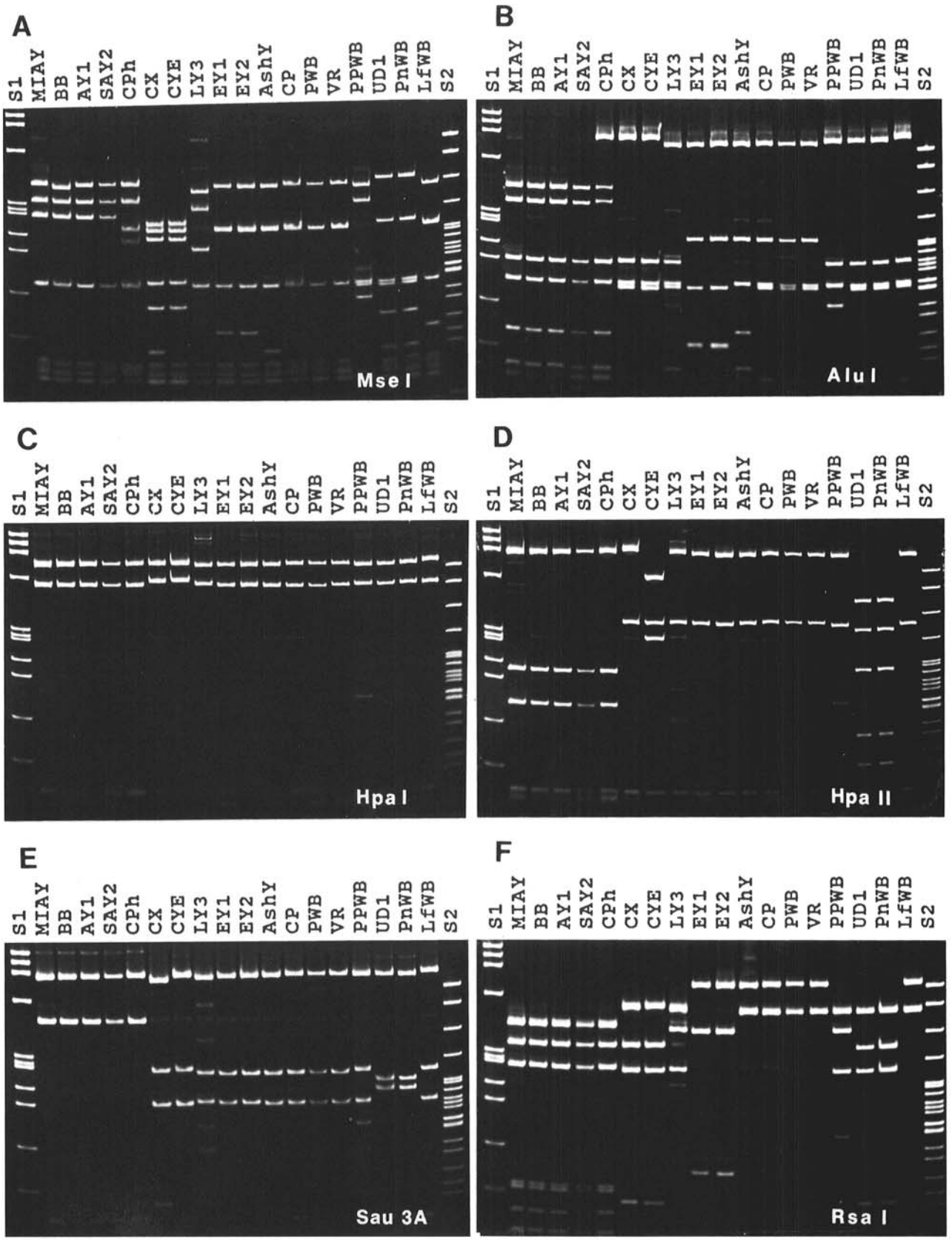

Fig. 3. Restriction fragment length polymorphism analyses of 16S rDNA amplified by polymerase chain reaction using primer pair R16F2R2 from representative mycoplasmalike organism (MLO) strains. DNA products were digested with restriction enzymes **A,** *Mse*I, **B,** *Alu*I, **C,** *Hpa*I, **D,** *Hpa*II, **E,** *Sau*3A, and **F,** *Rsa*I.

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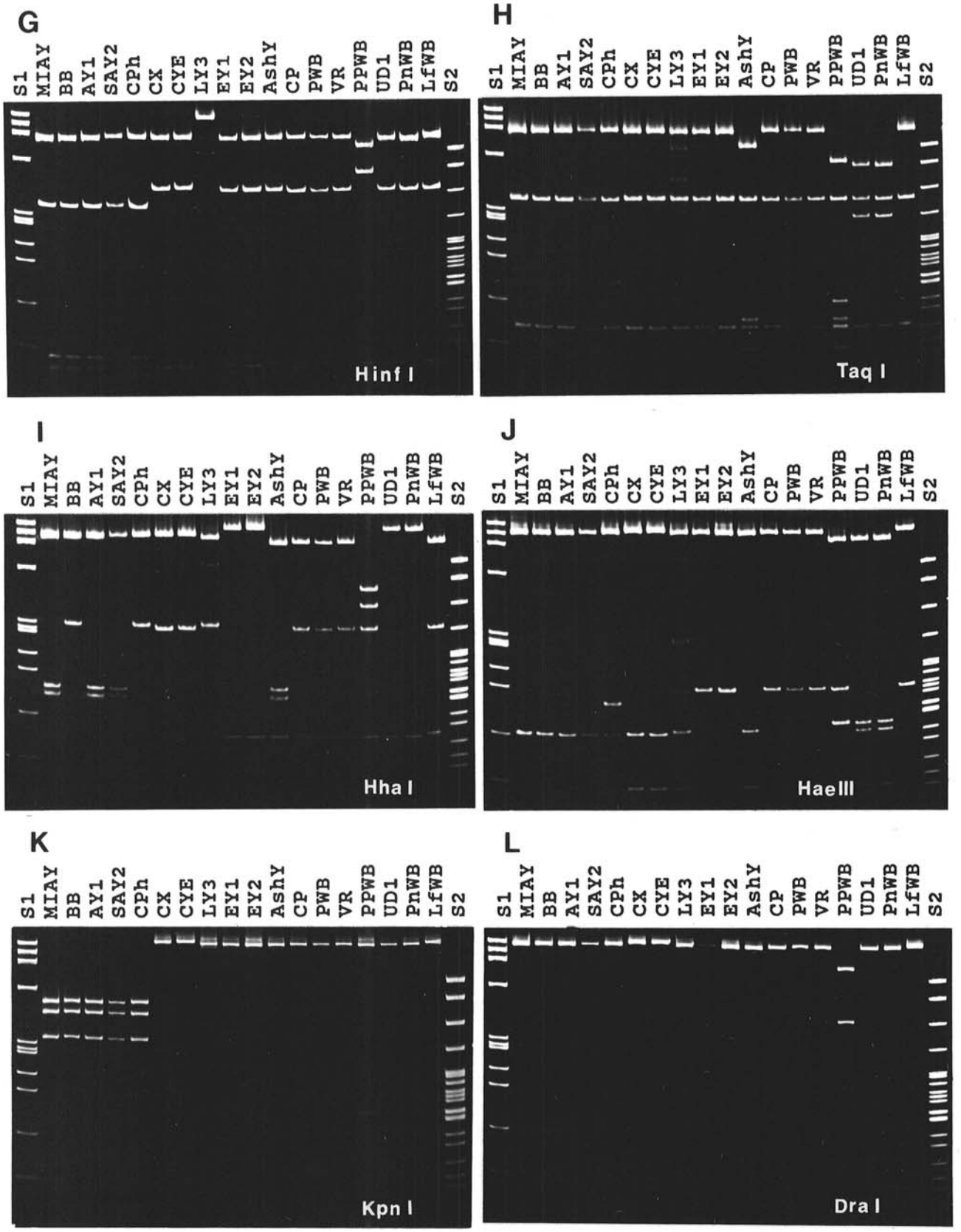

Fig. 3 (*continued*). Restriction fragment length polymorphism analyses of 16S rDNA amplified by polymerase chain reaction using primer pair R16F2R2 from representative MLO strains. DNA products were digested with restriction enzymes **G,** *Hin*fI, **H,** *Taq*I, **I,** *Hha*I, **J,** *Hae*III, **K,** *Kpn*I, and **L,** *Dra*I. Lanes S1 and S2, molecular weight markers are as described in Figure 2. All other abbreviations are defined in the text.

designated strain clusters. Similarity coefficients (F) derived by RFLP analysis ranged from 0.38 to 1.00 (Table 2). The F values for members of each previously designated MLO strain cluster were 0.93–1.00 for the AY MLO and the peach X MLO clusters and 1.00 for the CP MLO and the EY MLO strain clusters. A best-fit dendrogram, with a matrix correlation coefficient of 0.97, was obtained by cluster analysis of the similarity coefficients (Fig. 4). This analysis resulted in nine major 16S ribosomal RNA groups (16Sr) and 14 subgroups (Table 3). A tenth group containing apple proliferation MLOs from Italy (AP-A) and Germany (AP-G) were delineated in related work (Mogen, Lee, and Davis, *unpublished*). The group 16SrI was the largest. It contained 24 MLO strains including all the members of the AY MLO strain cluster, and several uncharacterized MLO strains (e.g., SL1, SL5, SL7, SL8, HyPh1, BBS1, and PaWB). Five subgroups were delineated within group 16SrI and two subgroups within group 16SrIII. The three subgroups determined by RFLP analysis, 16SrI-A, 16SrI-B, and 16SrI-C, coincided with the three subclusters (called types) previously designated within the AY MLO strain cluster (Table 3).

Classification of uncharacterized MLOs. Two MLOs associated with hydrangea phyllody (HyPh1) in Italy and blueberry stunt (BBS1) in the United States were chosen to test the classification system proposed in this work. 16S rDNA sequences of the two MLOs were amplified using the "universal" primer R16F2R2 and were analyzed by restriction digestion separately with 14 restriction enzymes (Fig. 5). In comparison with the representative MLO RFLP patterns shown in Figures 2 and 3, the collective 16S rDNA RFLP patterns of both HyPh1 and BBS1 MLOs were identified as belonging to group 16SrI. RFLP patterns resolved by restriction enzymes *Hha*I and *Hpa*II further classified the HyPh1 MLO as subgroup 16SrI-B and the BBS1 MLO as a new subgroup, 16SrI-E.

DISCUSSION

The inability to isolate MLOs in pure culture has long posed major problems in the classification and identification of these pathogens (13). The recent development of MLO-specific DNA probes has provided reliable means for identification of a number of MLOs and has made it possible, through nucleic acid hybridization and RFLP assays, to assess the genetic interrelatedness among those various MLOs (13). Although work of this kind has given rise to an MLO classification scheme in which distinct MLO clusters and subclusters have been recognized (13), the approach has limitations. Thus far, MLO-specific DNA fragments have been cloned from only a limited number of MLOs; stan-

TABLE 1. Summary of pattern groups produced by the restriction enzymes used for restriction fragment length polymorphism (RFLP) analysis of 16S rDNA from representative type strains of mycoplasmalike organisms (MLOs)

MLO strain[b]	16SrRNA group[c]	RFLP pattern type[a]														
		*Mse*I	*Alu*I	*Hpa*I	*Hpa*II	*Sau*3A	*Rsa*I	*Hin*fI	*Taq*I	*Hha*I	*Hae*III	*Kpn*I	*Dra*I	*Eco*RI	*Tha*I	*Eco*RII
BB	I-A	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1
AY1	I-B	1	1	1	1	1	1	1	1	2	1	1	1	1	1	1
MIAY	I-B	1	1	1	1	1	1	1	1	2	1	1	1	1	1	1
CPh	I-C	2	2	1	1	1	1	1	1	1	2	1	1	1	1	1
PaWB	I-D	3	1	1	1	1	1	1	1	2	1	1	1	1	1	1
BBS1	I-E	1	1	1	2	1	1	1	1	1	1	1	1	1	1	1
PnWB	II	4	3	1	3	2	2	2	2	3	2	2	1	1	1	1
CX	III-A	5	4	1	4	3	2	2	1	1	3	2	1	1	2	2
CYE	III-B	6	4	1	5	3	2	2	1	1	3	2	1	1	2	2
LY3	IV	7	5	1	4	3	3	3	1	1	3	2	1	1	2	2
EY1	V	8	6	1	4	3	4	2	1	3	4	2	1	1	2	3
CP	VI	9	7	1	4	3	5	2	1	4	4	2	1	1	2	3
AshY	VII	10	8	1	4	3	5	2	3	5	3	2	1	1	2	3
LfWB	VIII	8	4	1	4	3	5	2	1	4	4	2	1	1	1	3
PPWB	IX	11	9	1	4	3	6	4	4	6	5	2	2	1	1	3

[a] Numbers shown in each column represent distinct RFLP types with each restriction endonuclease.
[b] See Table 3 for descriptions of MLO strains.
[c] A, B, C, D, and E represent subgroups.

TABLE 2. Similarity coefficients, F[a], derived from restriction fragment length polymorphism (RFLP) analysis of 16S rDNA sequences of various mycoplasmalike organisms (MLOs)

	Representative strains of MLO exhibiting distinct RFLP patterns[b]															
	BBS1	MIAY	BB	AY1	CPh	PaWB	CX	CYE	LY3	EY1	AshY	CP	VR	PPWB	PnWB	LfWB
BBS1	1.00															
MIAY	0.95	1.00														
BB	0.97	0.97	1.00													
AY1	0.95	1.00	0.97	1.00												
CPh	0.94	0.96	0.95	0.96	1.00											
PaWB	0.94	0.99	0.96	0.99	0.93	1.00										
CX	0.50	0.50	0.50	0.50	0.50	0.50	1.00									
CYE	0.53	0.50	0.53	0.50	0.53	0.50	0.93	1.00								
LY3	0.57	0.55	0.57	0.55	0.55	0.54	0.73	0.72	1.00							
EY1	0.40	0.38	0.40	0.38	0.38	0.39	0.58	0.58	0.66	1.00						
AshY	0.44	0.47	0.44	0.47	0.42	0.45	0.58	0.59	0.64	0.75	1.00					
CP	0.44	0.42	0.44	0.42	0.42	0.41	0.64	0.67	0.73	0.88	0.84	1.00				
VR	0.44	0.41	0.44	0.41	0.41	0.41	0.64	0.67	0.72	0.87	0.83	0.99	1.00			
PPWB	0.43	0.42	0.43	0.42	0.39	0.40	0.45	0.47	0.60	0.53	0.60	0.60	0.59	1.00		
PnWB	0.51	0.49	0.51	0.49	0.47	0.48	0.53	0.54	0.49	0.45	0.44	0.47	0.47	0.48	1.00	
LfWB	0.46	0.46	0.46	0.46	0.44	0.45	0.67	0.67	0.71	0.76	0.68	0.83	0.81	0.60	0.51	1.00

[a] Each coefficient, F, is based on analyses of RFLP patterns generated by separate digestion of amplified 16S rDNA sequences with 15 different restriction enzymes.
[b] See Table 3 for descriptions of MLO strains.

dardized DNA probes useful for general detection and identification of numerous MLOs are not available. The present study demonstrated that a PCR-based procedure using a "universal" primer pair designed on the basis of 16S rRNA sequence of an MLO can be employed effectively to detect and identify a broad array of known and unknown MLOs from various plant hosts. By employing this primer pair in PCR, MLO-specific 16S rDNA sequences were first amplified from nucleic acid samples extracted from all 40 MLO-infected plants. No PCR products were obtained from samples extracted from healthy plants. Differentiation among and identification of these various MLOs were achieved by subsequent RFLP analyses of the amplified MLO 16S rDNA sequences by using a number of restriction enzymes.

Based on similarity coefficients (F) derived from RFLP analyses, the 40 MLO strains analyzed were differentiated and classified into nine distinct 16S rRNA groups and 14 subgroups (Fig. 4 and Table 3). Of the nine groups delineated, groups 16SrI, 16SrIII, 16SrV, 16SrVI, and 16SrVII coincided with the MLO strain clusters AY MLO, peach X-disease MLO, EY MLO, CP MLO, and AshY MLO, respectively, which were delineated previously on the basis of DNA sequence homology by nucleic acid hybridization assays using randomly cloned chromosomal DNA fragments (13). For example, group 16SrI consists of 15 MLO strains (Table 3), previously designated as members of the AY MLO strain cluster; group 16SrIII consists of three MLO strains (CX, WX, and CYE), previously designated as members of the peach X-disease MLO strain cluster; group 16SrV consists of three MLO strains, previously designated as members of the EY MLO strain cluster; group 16SrVI consists of two MLO strains, CP and PWB, previously designated as members of the CP MLO strain cluster; 16SrVII consists of the AshY MLO, previously designated as a member of the AshY MLO strain cluster. Furthermore, the delineation of subgroups within groups 16SrI and 16SrIII were essentially consistent with previous designation of types (subclusters) within the two corresponding MLO strain clusters (14,16). The results from analyses of 16S rDNA sequences in this study reaffirm the close phylogenetic relationships among members in each MLO strain cluster previously designated.

Fourteen distinct MLO 16S rDNA RFLP patterns (representing nine major 16S rRNA groups and 14 subgroups) were delineated collectively through analyses by 15 restriction enzymes. These reference RFLP pattern types (Table 1) were used as the basis for classification of unknown MLOs. Selection of "key" restriction enzymes for classification by use of RFLP analysis varies with the group of MLO. For instance, *Mse*I and *Alu*I were sufficient to classify MLOs in group 16SrI, but *Mse*I, *Alu*I, *Hpa*II, and

TABLE 3. Classification of mycoplasmalike organisms (MLOs) based on restriction fragment length polymorphism (RFLP) analyses[a] of their 16S rDNA sequences

MLO strain	MLO	Source	16S rRNA group[b]	Strain cluster (type)
BB	Tomato big bud	Arkansas	16SrI-A	AY MLO (I)
OKAY1	Western aster yellows	Oklahoma	16SrI-A	AY MLO (I)
AY27	Western aster yellows	Canada	16SrI-A	AY MLO (I)
CN13	Periwinkle little leaf	Connecticut	16SrI-A	AY MLO (I)
CN1	Periwinkle little leaf	Connecticut	16SrI-A	AY MLO (I)
NJAY	New Jersey aster yellows	New Jersey	16SrI-A	AY MLO (I)
NAY	Eastern aster yellows	Canada	16SrI-A	AY MLO (I)
AY1	Maryland aster yellows	Maryland	16SrI-B	AY MLO (II)
DAY	Western dwarf aster yellows	California	16SrI-B	AY MLO (II)
SAY2	Western severe aster yellows	California	16SrI-B	AY MLO (II)
TLAY2	Tulelake aster yellows	California	16SrI-B	AY MLO (II)
OKAY3	Western aster yellows	Oklahoma	16SrI-B	AY MLO (II)
CY2	Chrysanthemum yellows	Italy	16SrI-B	AY MLO (II)
NYAY	Eastern aster yellows	New York	16SrI-B	AY MLO (II)
CPh	Clover phyllody	Canada	16SrI-C	AY MLO (III)
MIAY	Michigan aster yellows	Michigan	16SrI-B	...[c]
SL1	Periwinkle yellows	Missouri	16SrI-A	...
SL5	Periwinkle yellows	Missouri	16SrI-B	...
SL7	Periwinkle yellows	Missouri	16SrI-B	...
SL8	Periwinkle yellows	Missouri	16SrI-B	...
HyPh1	Hydrangea phyllody	Italy	16SrI-B	...
BBS1	Blueberry stunt	Michigan	16SrI-E	...
IObWB	*Ipomoea obscura* witches'-broom	Taiwan	16SrI-B	...
PaWB	Paulownia witches'-broom	Taiwan	16SrI-D	...
PnWB	Peanut witches'-broom	Taiwan	16SrII	...
RBCWB	Red bird cactus witches'-broom	Taiwan	16SrII	...
UDI	...	Taiwan	16SrII	...
CX	Canada peach X-disease	Canada	16SrIII-A	Peach X MLO (I)
WX	Western X-disease	California	16SrIII-A	Peach X MLO (II)
CYE	Clover yellow edge	Canada	16SrIII-B	Peach X MLO (III)
LY3	Coconut lethal yellows	Florida	16SrIV	...
EY1	Elm yellows	New York	16SrV	EY MLO
EY2	Elm yellows	New York	16SrV	EY MLO
EYIta	Italy elm yellows	Italy	16SrV	EY MLO
CP	Clover proliferation	Canada	16SrVI	CP MLO
PWB	Potato witches'-broom	Canada	16SrVI	CP MLO
VR	Beet leafhopper-transmitted virescence agent	California	16SrVI	...
AshY	Ash yellows	New York	16SrVII	AshY MLO
LfWB	Loofah witches'-broom	Taiwan	16SrVIII	...
PPWB	Pigeon pea witches'-broom	Florida	16SrIX	...
AP-A	Apple proliferation	Italy	16SrX[d]	...
AP-G	Apple proliferation	Germany	16SrX[d]	...

[a]Restriction enzymes used for RFLP analysis: *Alu*I, *Mse*I, *Hpa*I, *Hpa*II, *Hha*I, *Kpn*I, *Eco*RI, *Eco*RII, *Dra*I, *Rsa*I, *Hin*fI, *Hae*III, *Sau*3A, *Taq*I, and *Tha*I.

[b]A, B, C, D, and E represent subgroups.

[c]Undesignated.

[d]This new group was designated on the basis of unpublished data (Mogen, Lee, and Davis).

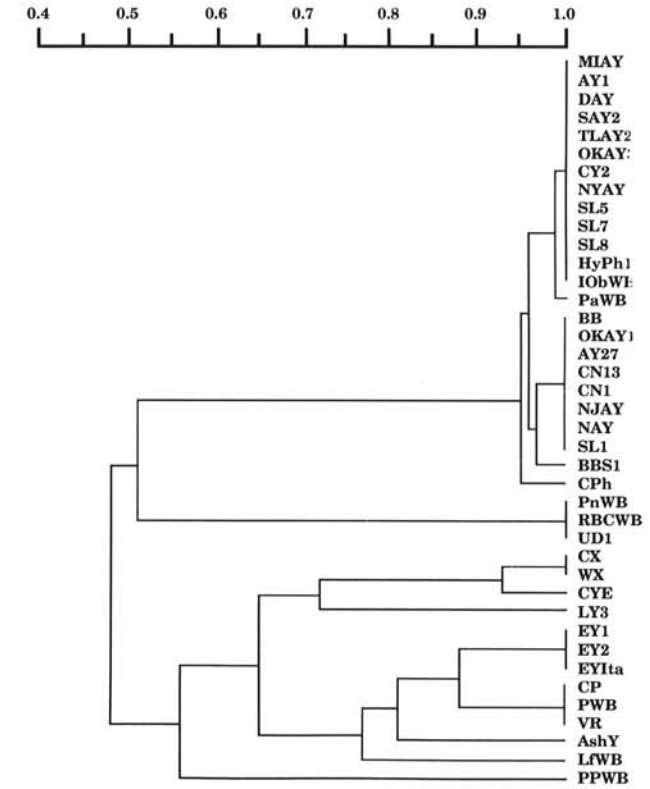

Fig. 4. Dendrogram obtained by cluster analysis of similarity coefficients, *F*, derived from restriction fragment length polymorphism analysis of 16S rDNA of the 40 mycoplasmalike organism (MLO) strains. Scale refers to similarity index. MLO strain descriptions are the same as in the text.

*Hha*I were needed for differentiation among members of this group. The restriction enzyme *Kpn*I was particularly useful to differentiate the 16SrI group from the other eight groups.

This classification system has proved very useful for the preliminary taxonomic affiliation (MLO 16S rRNA group and/or strain cluster designation) of a given unknown MLO and provides a basis for further characterization of the pathogen, which may require molecular probes (DNA or monoclonal antibodies) specifically designed for differentiation among members of a 16S rRNA group or strain cluster.

Analysis of 16S rRNA (or rDNA) sequences has been widely used to investigate phylogenetic relationships between microorganisms including Mollicutes (4,10,11,19,23). The phylogenetic relationships thus established have formed a reliable basis for classification of these microorganisms (4,23). Analysis of ribosomal gene sequences has often been carried out through direct sequencing (10,11,19,23). This can become very time-consuming when numerous strains must be analyzed. In the present work, extensive RFLP analyses of MLO 16S rDNA sequences were employed as an alternative means to assess the phylogenetic relationships among various MLOs. MLO 16S rRNA groups delineated by this approach coincided consistently with MLO strain clusters delineated previously on the basis of DNA hybridization assays using randomly cloned chromosomal DNA probes (5–7,11–16) and were in agreement with those achieved by direct sequencing (10). Thus, the approach using RFLP analyses of amplified 16S rDNA sequences provided a simple and quick alternative for differentiation and classification of uncultured MLOs. An uncharacterized MLO could be preliminarily classified and identified by comparison of its RFLP pattern type with known pattern types that represent designated MLO 16S ribosomal groups (Table 1). More than a dozen taxonomically unclassified MLOs from various sources have been identified in this work.

The present study not only illustrated detection of MLOs by amplification of 16S rDNA, it also presented a sensitive and rapid means for identification of these uncultured plant pathogens. The approach will facilitate phylogenetic study of many other unidentified MLOs and development of a comprehensive PCR-based classification system for MLOs.

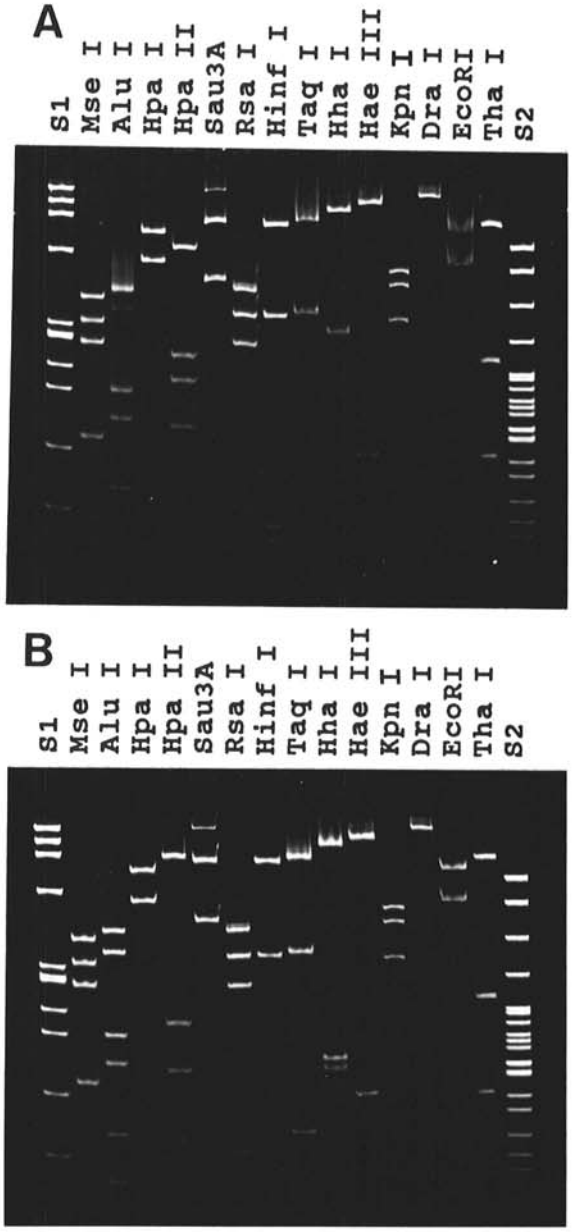

Fig. 5. Restriction fragment length polymorphism analyses of 16S rDNA amplified by polymerase chain reaction from BBS1 (**A**) and HyPh1 (**B**) mycoplasmalike organisms using primer pair R16F2R2. DNA products were digested with restriction enzymes as listed and electrophoresed through a 5% polyacrylamide gel. Lanes S1 and S2, molecular weight markers are as described in Figure 2.

LITERATURE CITED

1. Ahrens, U., and Seemüller, E. 1992. Detection of DNA of plant pathogenic mycoplasmalike organisms by a polymerase chain reaction that amplifies a sequence of the 16S rRNA gene. Phytopathology 82:828-832.
2. Chen, T.-A., Lei, J. D., and Lin, C. P. 1992. Detection and identification of plant and insect mollicutes. Pages 393-424 in: The Mycoplasmas. Vol. 5. R. F. Whitcomb and J. G. Tully, eds. Academic Press, New York.
3. Chiykowski, L. N., and Sinha, R. C. 1989. Differentiation of MLO disease by means of symptomatology and vector transmission. Zentralbl. Bakteriol. Hyg. (Suppl.) 20:280-287.

4. Cubeta, M. A., Echandi, E., Abernethy, T., and Vilgalys, R. 1991. Characterization of anastomosis groups of binucleate *Rhizoctonia* species using restriction analysis of an amplified ribosomal RNA gene. Phytopathology 81:1395-1400.
5. Davis, R. E., Lee, I.-M., Douglas, S. M., and Dally, E. L. 1990. Molecular cloning and detection of chromosomal and extrachromosomal DNA of the mycoplasmalike organism associated with little leaf disease in periwinkle (*Catharanthus roseus*). Phytopathology 80:789-793.
6. Davis, R. E., Prince, J. P., Hammond, R. W., Dally, E. L., and Lee, I.-M. 1992. Polymerase chain reaction detection of Italian periwinkle virescence mycoplasmalike organism (MLO) and evidence for relatedness with aster yellows MLOs. Petria 2:184-193.
7. Davis, R. E., Sinclair, W. A., Lee, I.-M., and Dally, E. L. 1991. Cloned DNA probes specific for detection of a mycoplasmalike organism associated with ash yellows. Mol. Plant-Microbe Interact. 5:163-169.
8. Deng, S., and Hiruki, C. 1991. Genetic relatedness between two nonculturable mycoplasmalike organisms revealed by nucleic acid hybridization and polymerase chain reaction. Phytopathology 81:1475-1479.
9. Deng, S., and Hiruki, C. 1991. Amplification of 16S rRNA genes from culturable and nonculturable mollicutes. J. Microbiol. Methods 14:53-61.
10. Kirkpatrick, B. C., Gao, J., and Harrison, N. 1992. Phylogenetic relationships of 15 MLOs established by PCR sequencing of variable regions within the 16S ribosomal RNA gene. (Abstr.) Phytopathology 82:1083.
11. Kuske, C. R., and Kirkpatrick, B. C. 1992. Phylogenetic relationships between the western aster yellows mycoplasmalike organism and other prokaryotes established by 16S rRNA gene sequence. Int. J. Syst. Bacteriol. 42:226-233.
12. Lee, I.-M., and Davis, R. E. 1988. Detection and investigation of genetic relatedness among aster yellows and other mycoplasmalike organisms by using cloned DNA and RNA probes. Mol. Plant-Microbe Interact. 1:303-310.
13. Lee, I.-M., and Davis, R. E. 1992. Mycoplasmas which infect plants and insects. Pages 379-390 in: Mycoplasmas: Molecular Biology and Pathogenesis. J. Maniloff, R. N. McElhansey, L. R. Finch, and J. B. Baseman, eds. American Society for Microbiology, Washington, DC.
14. 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.
15. Lee, I.-M., Davis, R. E., and Hiruki, C. 1991. Genetic relatedness among clover proliferation mycoplasmalike organisms (MLOs) and other MLOs investigated by nucleic acid hybridization and restriction fragment length polymorphism analyses. Appl. Environ. Microbiol. 57:3565-3569.
16. Lee, I.-M., Gundersen, D. E., Davis, R. E., and Chiykowski, L. N. 1992. Identification and analysis of a genomic strain cluster of mycoplasmalike organisms associated with Canadian peach (eastern) X disease, western X disease, and clover yellow edge. J. Bacteriol. 174:6694-6698.
17. Lee, I.-M., Hammond, R. W., Davis, R. E., and Gundersen, D. E. 1992. Phylogenetic relationships among plant pathogenic mycoplasmalike organisms (MLOs) based on 16S rRNA sequence analysis. (Abstr.) Phytopathology 82:1094.
18. Lee, I.-M., Mogen, B. D., and Davis, R. E. 1992. 16S rRNA primer pairs designed for general detection of and strain cluster identification among plant pathogenic mycoplasmalike organisms (MLOs) by PCR. (Abstr.) Phytopathology 82:1094.
19. Lim, P. O., and Sears, B. B. 1989. 16S rRNA sequence indicates that plant-pathogenic mycoplasmalike organisms are evolutionarily distinct from animal mycoplasmas. J. Bacteriol. 171:5901-5906.
20. McCoy, R. E., Caudwell, A., Chang, C. J., Chen, T. A., Chiykowski, L. N., Cousin, M. T., Dale, J., de Leeuw, G. T. N., Golino, D. A., Hackett, K. J., Kirkpatrick, B. C., Marwitz, R., Petzold, H., Sinha, R. H., Sugiura, M., Whitcomb, R. F., Yang, I. L., Zhu, B. M., and Seemüller, E. 1989. Plant diseases associated with mycoplasmalike organisms. Pages 545-560 in: The Mycoplasmas. Vol. 5. R. F. Whitcomb and J. G. Tully, eds. Academic Press, New York.
21. Nei, M., and Li, W.-H. 1979. Mathematical model for studying genetic variation in terms of restriction endonucleases. Proc. Natl. Acad. Sci. USA 76:5269-5273.
22. Schaff, D. A., Lee, I.-M., and Davis, R. E. 1992. Sensitive detection and identification of mycoplasmalike organisms by polymerase chain reactions. Biochem. Biophys. Res. Commun. 186:1503-1509.
23. Weisburg, W. G., Tully, J. G., Rose, D. L., Petzel, J. P., Oyaizu, H., Yang, D., Mandelco, L., Sechrest, J., Lawrence, T. G., Van Etten, J., Maniloff, J., and Woese, C. R. 1989. A phylogenetic analysis of the mycoplasmas: Basis for their classification. J. Bacteriol. 171:6455-6467.