Use of Mycoplasmalike Organism (MLO) Group-Specific Oligonucleotide Primers for Nested-PCR Assays to Detect Mixed-MLO Infections in a Single Host Plant

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ABSTRACT

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Oligonucleotide primer pairs R16(I)F1/R1, R16(III)F2/R1, and R16(V)F1/R1 for polymerase chain reactions (PCRs) were designed on the basis of mycoplasmalike organism (MLO) 16S rRNA sequences. The primer pair R16(I)F1/R1 specifically initiated amplification of 16S rDNA sequences among MLO strains in the MLO 16S rRNA group I, which includes aster yellows MLO and related strains; R16(III)F2/R1 specifically initiated amplification in the MLO 16S rRNA group III, which includes peach X-disease MLO and related strains; and R16(V)F1/R1 specifically initiated amplification in the MLO 16S rRNA group V, which includes elm yellows MLO and related strains. None of the primer pairs initiated amplification of 16S rDNA sequences from MLO strains in other groups or from other prokaryotes, including animal Mollicutes and plant pathogenic bacteria. An MLO group-specific primer pair allows sensitive detection and simultaneous classification of specific MLO strains from plant and insect sources. Nested-PCR assays using the universal primer pair R16F2/R2 and a group-specific primer pair further increased sensitivity in MLO detection. These specially designed assay procedures allowed for the first time detection of a secondary, cryptic MLO(s) associated with a single host plant.

Mycoplasmalike organisms (MLOs) are associated with diseases in several hundred plant species (25), but thus far none have been isolated in pure culture. Diseases have been termed according to host plants and symptoms induced in infected plants. In nature, MLOs are transmitted and spread by insect vectors. Generally, a given insect vector has its specificity in harboring an MLO(s) and has its preferential host plants (3,4,7,16). But it is not uncommon for an insect vector to carry more than one MLO strain and feed on more than one type of plant. Likewise, it is not uncommon for a given MLO strain to be transmitted by more than one insect vector into various plant species (3,4). The complexity of MLO ecology and the epidemiology of diseases that MLOs induce in nature may be attributed to the overlapping vector relationships and host ranges shared among various MLOs. The etiology of many uncharacterized MLO-induced diseases and the identification of associated MLOs have been emphasized in recent studies (2,25).

Molecular probes developed in recent years, e.g., MLO-specific monoclonal antibodies and cloned DNA probes, have made it possible to diagnose MLO-induced diseases by identifying the associated MLOs based on serological and DNA–DNA hybridization assays (1,2,8,12,15,18,19–22,30). These studies have already significantly expanded our knowledge in MLO ecology and changed the concepts about the etiology of MLO-associated diseases. It is now known that one type of MLO can cause diseases in both herbaceous and woody hosts, and that a given disease (e.g., grapevine yellows) can be caused by more than one type of MLO (8). Another long-suspected phenomenon, that a host plant or insect can be simultaneously infected by more than one type of MLO, has never been proven by these types of assays, however. The relatively limited sensitivity of these types of assays may account for the failure in detection of a secondary (minor) MLO(s) which may be present in very low titer in a host doubly or multiply infected with mixed types of MLOs.

The recent introduction of polymerase chain reaction (PCR) assays has greatly advanced the capacity to detect and identify MLOs. PCR assays using primer pairs designed on the basis of cloned MLO DNA fragments or MLO 16S rRNA gene sequences not only provide a very sensitive means of detecting a specific MLO strain, but also enable, for the first time, detection of a wide array of MLOs by use of a universal primer pair designed on the basis of MLO 16S rRNA gene sequence (1,9–13,15,20,22–24, 26–29,31). Through restriction fragment length polymorphism (RFLP) analyses of amplified MLO 16S rDNA sequences, MLOs have been classified into distinct MLO 16S rRNA groups and subgroups or MLO groups (23,29). It is now possible to detect and identify many previously uncharacterized MLOs associated with various hosts. However, we have found that universal primer pairs initiate amplification of 16S rDNA sequences from some other members of Mollicutes (e.g., *Acholeplasma* spp.) and prime amplification of nonspecific sequences from some insect vectors. Furthermore, universal primer pairs are not applicable for epidemiological studies where more than one type of MLO is associated with a given disease, or when a plant or insect host is doubly infected by a secondary MLO(s).

The objective of the present study was to design a series of MLO 16S rRNA group-specific primer pairs nested within the regions amplified by the universal primer pair R16F2/R2 developed previously (23). Nested-PCR assays using the universal primer pair and a selected MLO group-specific primer pair were employed to detect target MLOs associated with affected plant and insect vectors. This sensitive procedure was used to detect and identify the primary and, in some cases, a cryptic secondary MLO associated with several MLO-infected plants that were previously assumed to be infected by one MLO.

MATERIALS AND METHODS

Sources of mycoplasmalike organisms (MLOs) and other prokaryotes. Maryland aster yellows (AY1) was originally field collected in diseased periwinkle (*Catharanthus roseus* (L.) G. Don) at Beltsville, Maryland. 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: tomato big bud (BB) (J. Dale, University of Arkansas, Fayetteville); clover phyllody (CPh), clover yellow edge (CYE) in clover, and Canada peach X (CX) (L. N. Chiykowski, Agriculture Canada, Ottawa, Ontario, Canada); paulownia witches'-broom (PaWB), peanut witches'-broom (PnWB), and loofah (*Luffa* spp.) witches'-broom (LfWB) (H. J. Su and M. C. Tsai, National Taiwan University, Taipei, Taiwan); blueberry stunt (BBS1) in blueberry (D. C. Ramsdell, Michigan State University, East Lansing); palm lethal yellowing (LY3) (DNA sample from diseased coconut) and pigeon pea witches'-broom (PPWB) (N. A. Harrison, University of Florida, IFAS, REC, Ft. Lauderdale); elm yellows (EY1, EY2 [in Chinese elm]), ash yellows (AshY), and spirea (*Spiraea tomentosa* L.) stunt (SP1) (W. A. Sinclair, Cornell University, Ithaca, New York) (15); clover proliferation (CP) and potato witches'-broom (PWB) (C. Hiruki, University of Alberta, Edmonton, Alberta, Canada); Italy elm yellows (EYIta), apple proliferation (AP-A), and apricot chlorotic leaf roll in apricot (ACLR) (A. Bertaccini, Istituto di Patologia Vegetale, Bologna, Italy); and peach yellow leaf roll (PYLR) and western X-disease (WX) (B. C. Kirkpatrick, University of California, Davis). Several weed diseases found naturally infected with MLOs in Maryland—erigeron (*Erigeron canadensis* L., horseweed) yellows (ErY1, ErY2, ErY3, and ErY4), goldenrod (*Solidago* spp.) stunt (GS1 and GS2), lepidium (*Lepidium campestre* (L.) R. Br.) stunt (LS), oxalis (*Oxalis* spp.) yellows (OxY), and sowthistle (*Sonchus oleraceus* L.) stunt (SS)—were included in this study. Periwinkle plants and leafhoppers (*Macrosteles fascifrons* (Stål)) infected with Tulelake aster yellows (TLAY2) were provided by A. H. Purcell, University of California, Berkeley. DNA samples of *Acholeplasma laidlawii, Mycoplasma gallisepticum,* and *Mycoplasma capricolum* were provided by D. T. Kingsbury, George Washington University, Washington, D.C. Some representative plant pathogenic bacteria—*Pseudomonas syringae, Erwinia carotovora, Xanthomonas campestris* pv. *campestris,* and *Agrobacterium tumefaciens*—were provided by C.-H. Liao, USDA-ARS, Eastern Region Research Center, Philadelphia, Pennsylvania. Periwinkle plants infected with *Spiroplasma citri* were provided by G. N. Oldfield, University of California, Riverside.

Primer pairs and PCR conditions. The universal primer pair R16F2/R2 previously designed (23) on the basis of an AY MLO (24) was used in PCR assays for general detection of various MLOs. MLO 16S rRNA group-specific primer pairs R16(I)F1/R1, R16(III)F2/R1, and R16(V)F1/R1 were designed on the basis of unique sequences of MLO 16S rDNA in the region (about 1.2 kb) amplified with the universal primer pair R16F2/R2, among representative members of three MLO 16S rRNA groups, I, III, and V. These sequences were identified by direct PCR sequencing using standard dideoxy termination reactions (fmol kit, Promega Corporation, Madison, WI) followed by comparisons with sequences of corresponding regions of 16S rDNA from representative MLO strains in other MLO 16S rRNA groups (D. E. Gundersen, *unpublished*). A specific DNA fragment approximately 1.1 kb in size is amplified by using primer pairs R16(I)F1/R1 and R16(V)F1/R1; a fragment approximately 0.8 kb is amplified by primer pair R16(III)F2/R1. The oligonucleotide sequences of the three MLO group-specific primer pairs are:

1) R16(I)F1/R1:
   R16(I)F1, 5′-TAAAAGACCTAGCAATAGG-3′
   R16(I)R1, 5′-CAATCCGAACTGAGACTGT-3′

2) R16(III)F2/R1:
   R16(III)F2, 5′-AAGAGTGGAAAAACTCCC-3′
   R16(III)R1, 5′-TCCGAACTGAGATTGA-3′

3) R16(V)F1/R1:
   R16(V)F1, 5′-TTAAAAGACCTTCTTCGG-3′
   R16(V)R1, 5′-TTCAATCCGTACTGAGACTACC-3′

For PCR, total nucleic acid was extracted from healthy or MLO-infected plant or insect tissue, as described elsewhere (20). Nucleic acid extractions from elm tissues were performed as previously described (22). Nucleic acid samples were diluted in sterile deionized water to give a final concentration of 20 ng/μl. PCR assays were performed as previously described (28), with 20 ng each of total nucleic acid, 200 μM deoxynucleoside triphosphate (dNTP), and 0.4–1.0 μM primer pair. 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 the first cycle) denaturation step at 94 C, annealing for 2 min at 50 C, and primer extension for 3 min (10 min in final cycle) at 72 C. Tubes with the reaction mixture devoid of DNA templates 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.

Nested-PCR assays using universal and MLO 16S rRNA group-specific primer pairs. Nucleic acid samples extracted from AY1 MLO-, EY1 MLO-, and TLAY MLO-infected periwinkle plants and TLAY MLO-infected leafhoppers (*M. fascifrons*), as well as DNA or nucleic acid samples prepared from other MLOs representing 10 MLO 16S rRNA groups and 17 subgroups (23,29; I.-M. Lee, *unpublished*), and other prokaryotes as listed in Table 1, were used as templates for PCR assays. Included in the assays

TABLE 1. Summary of results from polymerase chain reaction (PCR) amplification of 16S rDNA from mycoplasmalike organisms (MLOs) and other prokaryotes

Template DNA	MLO 16S rRNA group and subgroup	16S rDNA amplification[a] using primer pair: R16 F2/R2	R16 (I) F1/R1	R16 (III) F2/R1	R16 (V) F1/R1
MLO strain					
BB	I-A	+	+	−	−
AY1	I-B	+	+	−	−
TLAY2	I-B	+	+	−	−
CPh	I-C	+	+	−	−
PaWB	I-D	+	+	−	−
BBS1	I-E	+	+	−	−
PnWB	II	+	−	−	−
CX	III-A	+	−	+	−
WX	III-A	+	−	+	−
CYE	III-B	+	−	+	−
LY3	IV	+	−	−	−
EY1	V	+	−	−	+
EY2	V	+	−	−	+
EYae2	V	+	−	−	+
EYIta1	V	+	−	−	+
EYIta2	V	+	−	−	+
CP	VI	+	−	−	−
AshY	VII	+	−	−	−
LfWB	VIII	+	−	−	−
PPWB	IX	+	−	−	−
AP-A	X-A[b]	+	−	−	−
ACLR	X-B[b]	+	−	−	−
Other prokaryotes					
Acholeplasma laidlawii		+	−	−	−
Mycoplasma gallisepticum		−	−	−	−
Mycoplasma capricolum		−	−	−	−
Spiroplasma citri		−	−	−	−
Pseudomonas syringae		+*	−	−	−
Xanthomonas campestris pv. *campestris*		−	−	−	−
Erwinia carotovora		−	−	−	−
Agrobacterium tumefaciens		−	−	−	−

[a] + = Specific PCR product, − = no detectable PCR product, * = nonspecific (smaller DNA fragment) PCR product.
[b] 16S rRNA X-A and -B (I.-M. Lee, *unpublished*).

were nucleic acid samples from TLAY MLO-infected periwinkle and insects, which had been artificially mixed with various amounts of EY MLO (strain EY1) nucleic acid. Primer pairs R16F2/R2, R16(I)F1/R1, and R16(V)F1/R1 were evaluated separately in the PCR assays for detection of MLOs. Specificities of the primer pairs R16(I)F1/R1 and R16(V)F1/R1 were further assessed by nested-PCR assays. In the nested-PCR assay, PCR products initially amplified using the universal primer pair R16F2/R2 were diluted (1/40) with sterile deionized water and used as template DNA for a subsequent series of 35 PCR cycles in which reaction mixtures contained either specific primer pair R16(I)F1/R1 or R16(V)F1/R1.

Nested-PCR assays for detection of MLOs associated with naturally infected plant hosts. Nested-PCR assays using the universal primer pair R16F2/R2 followed by the use of one of the three MLO group-specific primer pairs (R16(I)F1/R1, R16(III)F2/R1, or R16(V)F1/R1) were employed to detect MLO(s) associated with some previously known diseases (PYLR, PWB, CYE, and SP1) in crops or weeds, and unknown MLO diseases in the weeds collected in the field.

RFLP analyses of PCR products. MLO 16S rDNA sequences amplified by PCR using the primer pairs R16F2/R2, R16(I)F1/R1, R16(III)F2/R1, and R16(V)F1/R1 were analyzed by restriction endonuclease digestion. Between 5 and 10 μl of each PCR product was digested separately with two selected restriction endonucleases, *Alu*I (GIBCO BRL, Gaithersburg, MD) and *Mse*I (New England Biolabs, Beverly, MA) (23). For analyses of nested-PCR products amplified from naturally infected plant hosts, four

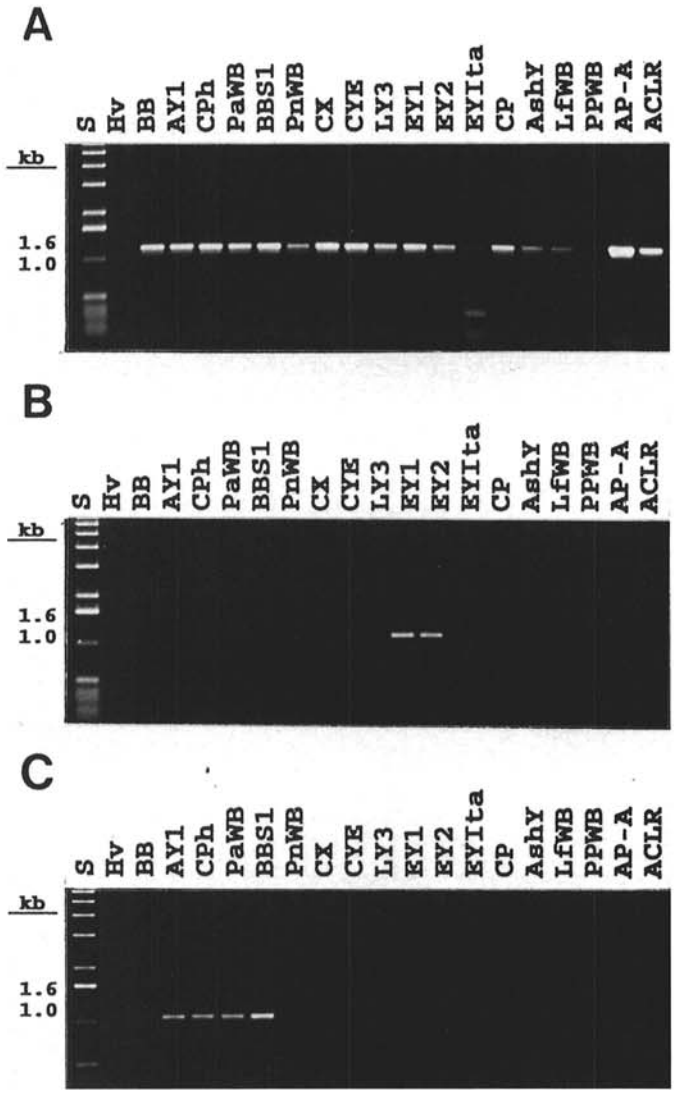

Fig. 1. Polymerase chain reaction (PCR) amplification of a 16S rDNA sequence from various mycoplasmalike organisms (MLOs) representing 10 MLO 16S rRNA groups and 17 subgroups using primer pairs **A,** R16F2/R2, **B,** R16(V)F1/R1, and **C,** R16(I)F1/R1. PCR products (35 cycles) were separated by electrophoresis through a 1% agarose gel. Lane S, 1-kb DNA ladder (GIBCO BRL); lane Hv, healthy *Catharanthus roseus*. Other abbreviations are as described in text.

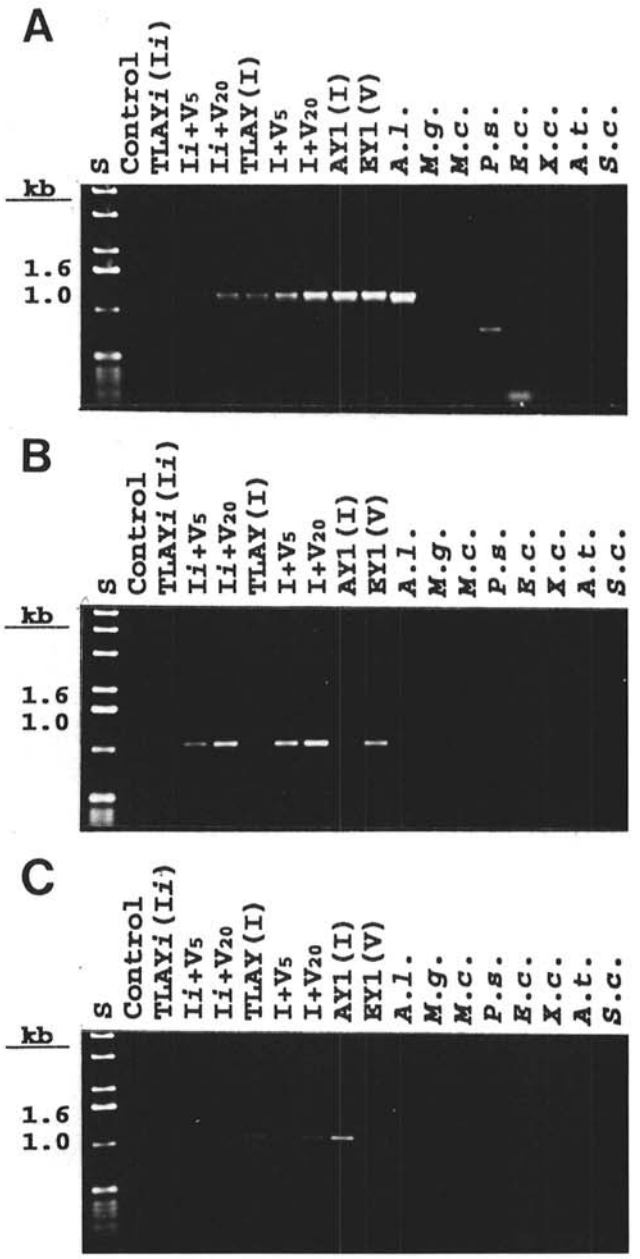

Fig. 2. Polymerase chain reaction (PCR) amplification of a 16S rDNA sequence from an individual mycoplasmalike organism (MLO) strain, mixed MLOs, or from some representative Mollicutes and plant pathogenic prokaryotes, using primer pairs **A,** R16F2/R2, **B,** R16(V)F1/R1, and **C,** R16(I)F1/R1. PCR products (35 cycles) were separated by electrophoresis through a 1% agarose gel. Lane S, 1-kb DNA ladder (GIBCO BRL); lane Control, water; TLAYi, TLAY MLO-infected insects (*Macrosteles fascifrons*); TLAY, AYI, and EY1: TLAY MLO-, AY1 MLO-, and EY1 MLO-infected *Catharanthus roseus*; I and V, MLO 16S rRNA group I and V; *A.l., Acholeplasma laidlawii*; *M.g., Mycoplasma gallisepticum*; *M.c., M. capricolum*; *P.s., Pseudomonas syringae*; *E.c., Erwinia carotovora*; *X.c., Xanthomonas campestris* pv. *campestris*; *A.t., Agrobacterium tumefaciens*; *S.c., Spiroplasma citri*. I(or Ii)+V represents mixed MLOs consisting of TLAY strain (MLO 16S rRNA group I) from plant (I) or insect (Ii) source and EY1 strain (group V) from *C. roseus*. Two different quantities of EY1 MLO nucleic acid samples (V_5 = 5 ng, V_{20} = 20 ng) were mixed with 20 ng of TLAY MLO nucleic acid sample extracted from plant or insects.

restriction endonucleases were employed: *Alu*I, *Mse*I, *Hpa*II, and *Hha*I(GIBCO BRL). The restriction products were then separated by electrophoresis through a 5% polyacrylamide gel followed by staining in ethidium bromide and visualization of DNA bands using a UV transilluminator.

RESULTS

MLO 16S rRNA group-specific primer pairs for PCR. The universal primer pair, R16F2/R2, primed amplification of 16S rDNA sequences from all MLOs tested (Table 1, Fig. 1A) and some Mollicutes, e.g., *A. laidlawii.* It also primed amplification of a nonspecific DNA sequence from *P. syringae* (Fig. 2A). The primer pair R16(V)F1/R1, derived from 16S rDNA sequence of the EY1 MLO, primed amplification of 16S rDNA sequences from American strains of the EY MLO (strains EY1, EY2, and EYae2), as well as the Italian EY MLO strains, EYIta1 and EYIta2 (Table 1, Fig. 1B). However, the primer pair did not prime amplification of DNA from any other MLO that did not belong to the EY MLO strain cluster or to MLO 16S rRNA group V (Table 1, Fig. 1B). The primer pair R16(I)F1/R1 primed amplification of 16S rDNA sequences from the five MLO strains, AY1, BB, CPh, PaWB, and BBS1, representing five subgroups of the MLO 16S rRNA group I (Table 1, Fig. 1C). It did not prime amplification of DNA from any other MLO not belonging to MLO 16S rRNA group I (Table 1, Fig. 1C). The primer pair R16(III)F2/R1 primed amplification of 16S rDNA sequences from the three MLO strains, CX, WX, and CYE, representing two subgroups of the MLO 16S rRNA group III (Table 1). None of the three primer pairs R16(I)F1/R1, R16(III)F2/R1, and R16(V)F1/R1 primed amplification of DNA from other representative Mollicutes from animal sources (*A. laidlawii, M. gallisepticum,* and *M. capricolum*), nor did any of the three prime amplifications of 16S rDNA sequences from any of several representative plant pathogenic prokaryotes, e.g., *P. syringae, X. c. campestris, E. carotovora, A. tumefaciens,* and *S. citri* (Table 1, Fig. 2B and C).

Specific detection of target MLOs from plant and insect sources. Nucleic acid extracts from a periwinkle plant and an insect vector (*M. fascifrons*) infected with an AY MLO strain (TLAY2) were mixed with various amounts of nucleic acid extract from EY MLO (strain EY1) infected periwinkle. The mixed nucleic acid extracts were used in PCR assays using the three primer pairs, R16F2/R2, R16(V)F1/R1, and R16(I)F1/R1. While the universal primer pair R16F2/R2 primed amplification of MLO 16S rDNA sequences from every sample containing MLO DNA templates (Fig. 2A), the primer pair R16(V)F1/R1 primed amplification only from samples containing the EY MLO DNA templates (Fig. 2B) and the primer pair R16S(I)F1/R1 primed amplification only from samples containing the TLAY2 MLO DNA templates (Fig. 2C).

When employed to reamplify (nested-PCR assay) the PCR products (MLO 16S rDNA sequences) initially amplified with the universal primer pair R16F2/R2 (Fig. 3A), the primer pair R16(V)F1/R1 only primed amplification of PCR products initially amplified from samples containing the EY MLO DNA (Fig. 3B), while the primer pair R16(I)F1/R1 only primed amplification of PCR products initially amplified from samples containing the TLAY MLO (Fig. 3C).

RFLP analyses of MLO 16S rDNA sequences. RFLP analyses of the amplified PCR products using two selected restriction enzymes, *Alu*I (Fig. 4A and B) and *Mse*I (Fig. 4C and D), confirmed specificities of primer pairs R16(V)F1/R1 and R16(I)F1/R1. Analyses of the PCR products amplified with primer pair R16F2/R2 resulted in three collective 16S rDNA RFLP pattern types (Fig. 4Aa and Ca). Pattern types characteristic of the AY MLO or MLO 16S rRNA group I (23) were obtained with the AY1 (Fig. 4Aa and Ca, lanes 1 and 3) or TLAY2 MLO DNA as templates, whereas pattern types characteristic of the EY MLO or MLO 16S rRNA group V (23) were obtained with the EY MLO DNA as templates (Fig. 4Aa and Ca, lane 2). When mixed MLO (EY and AY) DNA was used as a template, a third pattern that was clearly a composite of both AY and EY MLO RFLP patterns was obtained (Fig. 4Aa and Ca, lanes 5–8). Nonspecific banding patterns (not characteristic of either AY or EY MLOs) were obvious when PCR products amplified with template DNAs from MLO-infected insect sources were analyzed (Fig. 4Aa and Ca, lanes 4, 7, and 8).

In contrast, analyses of the PCR products amplified with the primer pair R16(V)F1/R1 resulted in only one collective RFLP pattern type that was characteristic of the EY MLO (Fig. 4Ab and Cb, lanes 1–4). This primer pair specifically primed amplification of the EY MLO 16S rDNA sequence from nucleic acid samples prepared from TLAY MLO-infected plants or

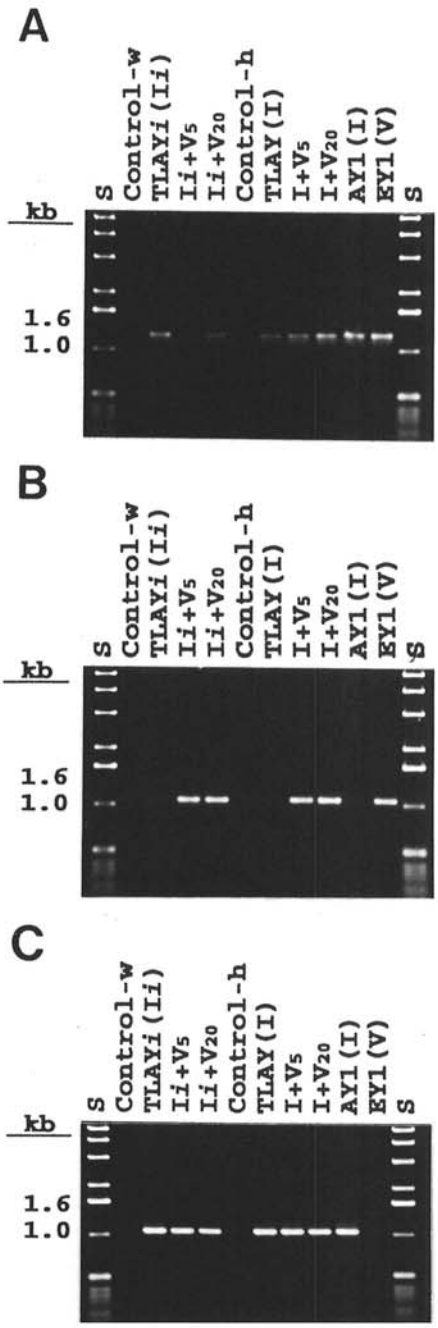

Fig. 3. Direct and nested-PCR amplification of a 16S rDNA sequence from an individual MLO strain or mixed MLOs. Direct PCR amplification **A,** was performed (35 cycles) using the universal primer pair R16F2/R2, while nested-PCR amplifications were performed initially using primer pair R16F2/R2 for 35 cycles followed by second PCR amplification (35 cycles) using MLO group-specific primer pairs **B,** R16(V)F1/R1 and **C,** R16(I)F1/R1, repectively. PCR products were separated by electrophoresis through a 1% agarose gel. Lane S, 1-kb DNA ladder; lane Control-w, water; lane Control-h, healthy *Catharanthus roseus.* Other abbreviations are as described in Figure 2.

insects, to which EY MLO DNA templates (EY1 MLO) had been added. Analyses of MLO 16S rDNA sequences resulting from reamplification with the primer pair R16(V)F1/R1, again depicted a single RFLP pattern type characteristic of the EY MLO (Fig. 4Ac and Cc, lanes 1–4), indicating amplification of only the EY MLO 16S rDNA sequences. Likewise, analyses of the PCR products amplified with the primer pair R16(I)F1/R1 resulted in only one collective RFLP pattern type that was characteristic of the AY MLO or MLO 16S rRNA group I (Fig. 4Bb and Db, lanes 1–4). Analyses of MLO 16S rDNA sequences resulting from reamplification with primer pair R16(I)F1/R1 also resulted in the RFLP pattern type characteristic of the AY MLO (Fig. 4Bc and Dc, lanes 1–5). Substantially more specific PCR products were yielded by reamplification using the nested-PCR procedures than by direct amplification with primer pair R16(I)F1/R1 (Fig. 4Bb and Db) or R16(V)F1/R1 (Fig. 4Ab and Cb). The banding patterns in Figure 4Bb and Db could be intensified by applying increased amount of digested PCR products (10–15 μl) (data not

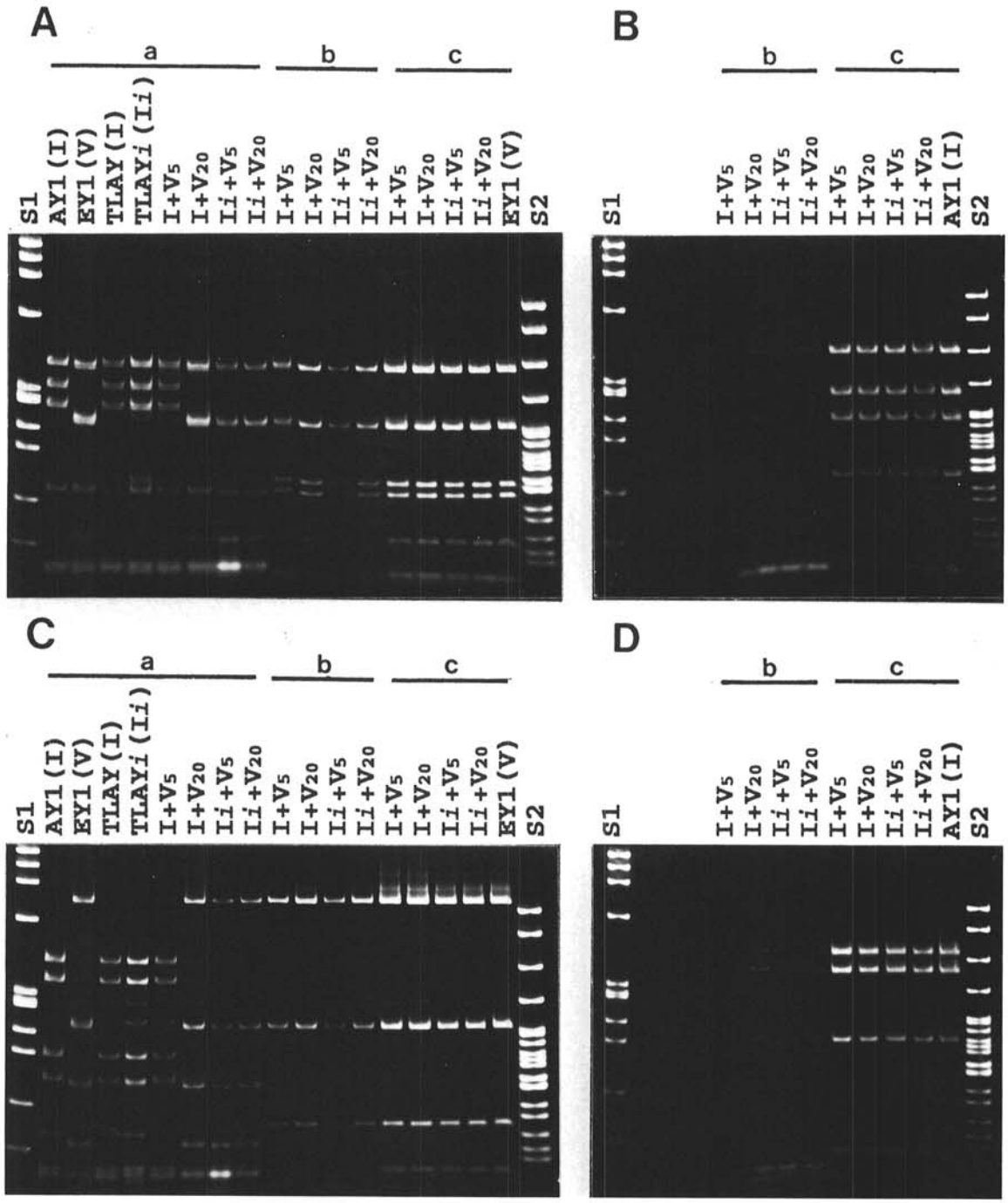

Fig. 4. Restriction fragment length polymorphisms of MLO 16S rDNA amplified by polymerase chain reaction (PCR) using the universal primer pair, MLO 16S rRNA group-specific primer pairs, and nested primer pairs with a combination of the universal and an MLO group-specific pair. DNA products were digested with restriction enzymes (**A** and **B,** *Mse*I; **C** and **D,** *Alu*I) and separated by electrophoresis through a 5% polyacrylamide gel. Bars Aa and Ca, PCR products amplified using the universal primer pair R16F2/R2; bars Ab and Cb, PCR products amplified using MLO 16S rRNA group V-specific primer pair R16(V)F1/R1; bars Bb and Db, PCR products amplified using MLO 16S rRNA group I-specific primer pair R16(I)F1/R1; bars Ac and Cc, nested-PCR products amplified using the primer pair R16F2/R2 followed by reamplification with primer pair R16(V)F1/R1; bars Bc and Dc, nested-PCR products amplified using the primer pair R16F2/R2 followed by reamplification with primer pair R16(I)F1/R1. Lane S1, ϕX174 RF I DNA *Hae*III digest, fragment sizes in base pairs from top to bottom are 1,353, 1,078, 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 are 622, 527, 404, 307, 242, 238, 217, 201, 190, 180, 160, 147, 123, 110, 90, 76, 67, 34, 26, 15, and 9. Other abbreviations same as described in Figures 2 and 3.

shown).

Cryptic secondary MLO(s) detected by nested-PCR assays. Direct PCR assays using universal primer pair R16F2/R2 (Fig. 5A) or group-specific primer pairs (data not shown) detected the primary MLO associated with many of the diseases examined. Primary MLOs associated with these diseases were identified and classified (MLO 16S rRNA group affiliation) (23) as: PYLR (group III-A), CYE (group III-B), PWB (group VI), SP1 (group III-A), ErY1 (group I-B), ErY2 (group I-B), ErY3 (group I-B), ErY4 (group I-A), GS1 (group I-A), and GS2 (group I-A) (Table 2). Direct PCR assays failed to detect MLOs associated with diseases LS, OxY, and SS (Fig. 5A). Nested-PCR assays using a combination of the universal primer pair and a group-specific pair detected the presence of a cryptic primary MLO associated with weed diseases LS, OxY, and SS and secondary MLO(s) associated with diseases PYLR, CYE, and PWB and weed diseases SP1, ErY4, and GS1 (Fig. 5B and C). Cryptic MLOs associated with these diseases were identified as: PYLR (group I-B), CYE (group I-B), PWB (group I-B), SP1 (Group I-C), ErY4 (group III-A), and GS1 (group III-A) (Table 2). MLO 16S rRNA group affiliation of each MLO detected was confirmed by RFLP analysis of the amplified 16S rDNA sequence (data not shown).

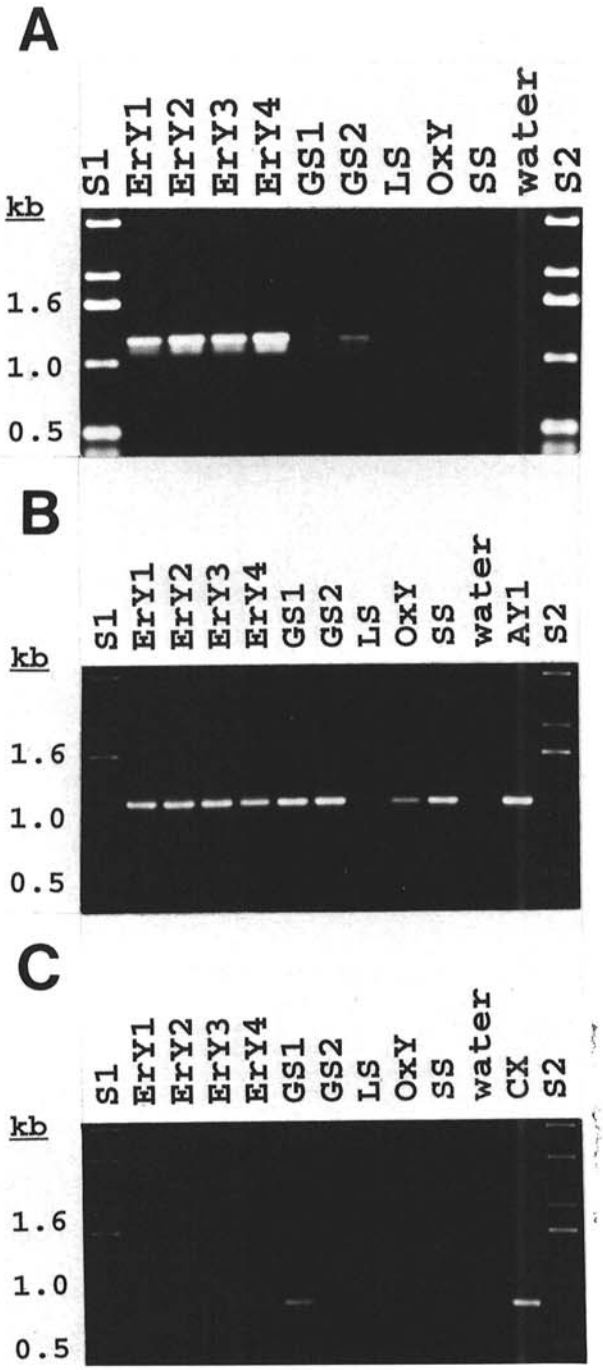

Fig. 5. Direct and nested–polymerase chain reaction (PCR) amplification of mycoplasmalike organism (MLO) 16S rDNA sequences from naturally infected weeds. Direct PCR amplification **A,** was performed (35 cycles) using the universal primer pair R16F2/R2, while nested-PCR amplifications were performed initially using primer pair R16F2/R2 for 35 cycles and followed by second PCR amplification (35 cycles) using MLO group-specific primer pair **B,** R16(I)F1/R1 and **C,** R16(III)F2/R1, respectively. PCR products were separated by electrophoresis through a 1% agarose gel. Lanes S1 and S2, 1-kb DNA ladder; lane water, water control. Other abbreviations same as described in Table 2.

DISCUSSION

Mycoplasmalike organisms, which are apparently confined to the phloem tissues in infected plants, can be present in relatively low titers and are often unevenly distributed among the plant host organs. Highly sensitive means are required for accurate diagnosis of MLO-induced plant diseases. Sensitive MLO detection is important in screening for disease-free planting stocks and in identification of potential insect vectors that carry MLOs. The PCR assay, in which copies of target DNA fragments present in samples are extensively amplified, may provide one of the most sensitive means for detection of this type of pathogen.

PCR assays using primer pairs designed on the basis of the sequence of cloned EY, AY, or other MLO DNA fragments have

TABLE 2. Mixed mycoplasmalike organisms (MLOs) detected by nested-polymerase chain reaction (PCR) assays in greenhouse-maintained and field-collected host plants

MLO disease (source)	MLOs identified		
	16S rRNA group I	16S rRNA group III	16S rRNA group V
Peach yellow leaf roll, PYLR (in periwinkle)	I-B	III-A[a]	–
Clover yellow edge, CYE (in clover)	I-B	III-B[a]	–
Potato witches'-broom, PWB (in periwinkle)[a]	I-B	–	–
Spirea stunt, SP1 (in periwinkle)	I-C	III-A[a]	–
Erigeron yellows, ErY1 (in horseweed)	I-B[a]	–	–
Erigeron yellows, ErY2 (in horseweed)	I-B[a]	–	–
Erigeron yellows, ErY3 (in horseweed)	I-B[a]	–	–
Erigeron yellows, ErY4 (in horseweed)	I-A[a]	III-A	–
Goldenrod stunt, GS1 (in goldenrod)	I-A[a]	III-A	–
Goldenrod stunt, GS2 (in goldenrod)	I[a]	–	–
Lepidium stunt, LS (in lepidium)	I[a]	–	–
Oxalis yellows, OxY (in oxalis)	I[a]	–	–
Sowthistle stunt, SS (in sonchus)	I[a]	–	–

[a]The primary MLO associated with each of the diseases was identified by PCR assays using the universal primer R16F2/R2 followed by restriction fragment length polymorphism analyses of the amplified 16S rDNA sequences, or by PCR assays using MLO group-specific primer pairs. Primary MLO associated with PWB is potato witches'-broom MLO that has been classified as a member of MLO 16S rRNA group VI (23).

been employed effectively to detect EY, AY, or other MLO strains associated with affected plant hosts (10,12,20,22,27,28). However, such primers may not amplify DNA of all strains of an MLO. For example, primer pairs derived from a cloned EY MLO DNA fragment failed to detect some EY MLO variants present in Italy, which have been shown to be closely related to the American EY MLO strains based on dot hybridization analysis (22). This type of primer is less applicable for use in epidemiological study of disease. Recently, a "universal" primer pair (R16F2/R2) derived from conserved regions of the 16S rRNA sequence of an AY MLO strain was employed successfully to detect a wide array of MLOs, including the AY and EY MLO variants from plant sources (23). But this primer pair could also prime amplification of 16S rDNA or other nonspecific DNA sequences from some Mollicutes and other prokaryotes (Fig. 2A). In some cases, MLO 16S rDNA together with other nonspecific DNA sequences was amplified (Fig. 2A) from some plant (Fig. 1A) or insect (Fig. 4A, lane 5) hosts.

In the present study, moderately reactive primer pairs R16(I)F1/R1, R16(III)F2/R1, and R16(V)F1/R1 for PCR were developed based on the 16S rDNA sequences that are characteristic of MLO 16S rRNA groups I, III, and V, respectively. In contrast to the universal primer pair R16F2/R2, the primer pairs R16(I)F1/R1, R16(III)F2/R1, and R16(V)F1/R1 primed amplification of 16S rDNA sequences only from MLO strains that are members of the MLO 16S rRNA group I, group III, and group V, respectively. RFLP analyses of the amplified 16S rDNA sequences confirmed and validated the specificity of these primer pairs. The present study demonstrated that a given MLO group-specific primer pair can be employed to prime specific amplification of MLO 16S rDNA sequences of interest from plant as well as insect samples containing artificially mixed MLOs. The primer pair R16(V)F1/R1 has been used successfully to specifically detect EY MLO from another insect vector, *Euscelidius variegatus* (Kirshbaum) (J. P. Prince, R. E. Davis, and I.-M. Lee, *unpublished*). The nested-PCR assays using the universal primer pair R16F2/R2 and a group-specific primer pair further increased the sensitivity of MLO detection. The novel nested-PCR assays allow simultaneous amplification of 16S rDNA sequences from all associated MLOs and subsequent detection of a selected target MLO from an insect vector and plant hosts, and for the first time, demonstrated that in nature a plant can be infected simultaneously by more than one type of MLO.

Pioneering works conducted by Kunkle and others (3–7,14,16, 17) on host range and insect vector specificity have provided the fundamental concept that depicts the complexity of MLO ecology. MLOs are associated with both plants and insect vectors. Because of overlapping vectors and plant host ranges shared among MLOs, it has been long suspected that a host plant or insect can be infected by more than one type of MLO. Mixed infections by a primary and secondary MLO(s), however, have never been demonstrated. Preliminary data obtained from the present study indicated that association of more than one kind of MLO with a single host plant commonly occurs, although the secondary MLO(s) is usually present at very low titer. Mixed infections occur in cultivated crops as well as in weed hosts.

It is difficult to assess the significance of the role of secondary MLOs in pathogenesis of a given disease. This may depend on the cell titer of the secondary MLO(s) present in the host plant. Earlier studies showed that each MLO has its preferential plant or insect hosts (5,7) and may not propagate well in nonhosts. Nevertheless, these secondary MLOs appear to be readily transmissible by a "nonvector," or nonpreferential vector, along with the primary MLO. When a mixed infection occurs in a plant host, the cell titer of the secondary MLO may build up to a level that will interfere with disease development and subsequently modify expression of symptoms or syndrome in the host.

The new perspective on mixed MLO infections may lead to redefining the etiologies of many MLO-associated diseases. This is especially true for those diseases associated with perennial plants, whose long life spans provide vast opportunities for different insect vectors bearing MLOs to visit over time. The constituent MLO(s) associated with a perennial host plant may vary seasonally or yearly. The primary MLO associated with a host plant at one season or in one particular year may become a secondary one at another season or in another year. It may be difficult to determine which MLO is the primary pathogen for a disease associated with a perennial host.

The recent development of PCR-based assays for detection and classification of MLOs has advanced our knowledge on etiologies of numerous MLO-associated diseases worldwide. It is known that an MLO or its closely related strains can be associated with diseases in many more plant species than previously thought, and that a given disease can be caused by different MLOs in different geographical regions (27). The nested-PCR assays using a combination of universal and MLO group-specific primer pairs have further advanced our capability to detect secondary or cryptic MLOs that were previously undetected. This type of assay should particularly facilitate phytosanitary screening processes where a requirement of absolutely pathogen-free plant materials is essential, and facilitate epidemiological studies of MLO-induced diseases associated with woody plant hosts, where MLOs are often present in unusually low titer.

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