Molecular Detection of Diverse Mycoplasmalike Organisms (MLOs) Associated with Grapevine Yellows and Their Classification with Aster Yellows, X-Disease, and Elm Yellows MLOs

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ABSTRACT

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Polymerase chain reactions (PCR) and restriction analyses of PCR-amplified DNA were used to detect and differentiate strains of mycoplasmalike organisms (MLOs) associated with grapevine yellows detected in naturally diseased grapevines in the United States and Italy. At least three major groups of grapevine-infecting MLOs were delineated. FDVA1 MLO, discovered in yellows-diseased grapevines in Virginia, and *flavescence dorée* MLO strain FDU from northern Italy were classified with X-disease MLOs; grapevine yellows-associated MLO strains FDG from Germany, CA1, CH1, SAN1, and SAN2 from northern Italy, and FDB and FDR from southern Italy were classified with aster yellows MLOs; and *flavescence dorée* MLO strain FDF from France was classified in the elm yellows MLO group. The data support consideration of grapevine yellows as a complex of several diseases of diverse etiologies.

Additional keywords: disease diagnosis, epidemiology, indexing, Mollicutes, *Vitis*.

Grapevine yellows diseases characterized by similar symptoms have been reported in several countries including France, Italy, Germany, the United States, and Australia (reviewed in 8). These diseases are among the most serious known in grapevine (6,7). The causal pathogens are believed to be mycoplasmalike organisms (MLOs) transmitted to grapevine in parts of Europe by *Scaphoideus titanus* Ball, a leafhopper introduced into Europe from North America (5,8,36). In France, two grapevine yellows diseases, *flavescence dorée* and *bois noir,* have been distinguished in part on the basis of transmission of the *flavescence dorée* pathogen by *S. titanus* and the inability of this insect to transmit the *bois noir* pathogen (9). Two grapevine yellows diseases may also occur in Italy. *S. titanus* is found in northern Italy and is apparently a vector of the northern Italy *flavescence dorée* pathogen. In southern Italy, *S. titanus* has not been reported, and a vector of the southern European grapevine yellows pathogen has not been identified (8,31).

Biological criteria have long been used in detection, differentiation, and characterization of grapevine yellows diseases because it has not been possible to isolate the pathogens in pure culture. Symptoms exhibited by naturally infected plants, production of characteristic symptoms in graft-inoculated plants, identity of insect vectors capable of transmitting the pathogens, and characteristics of disease spread have served as bases for comparison with the *flavescence dorée* disease originally reported in France (4,8,10). New DNA-based and serological methods provide important means for rapid, sensitive, and accurate detection and characterization of MLOs associated with grapevine yellows (14,15,18,29). DNA dot hybridizations and restriction fragment length polymorphism (RFLP) analysis of chromosomal DNA permit detection and recognition of distinctions between MLOs associated with *flavescence dorée* in northern Italy and those associated with southern European grapevine yellows in southern Italy (15).

We have studied 11 MLO strains associated with grapevine yellows diseases in France, Italy, Germany, and the United States. In this work, we investigated detection of these MLOs and their relationships to other MLOs that had been classified earlier in a system of strain clusters (23). Previous work in our laboratory and in others has demonstrated sensitive detection and differentiation of MLOs through the use of polymerase chain reactions (PCR) (17,20,28,34). Oligonucleotide primers for PCR have been designed on the basis of nucleotide sequences determined for well-characterized, randomly cloned DNA fragments and for 16S rRNA gene sequences, making possible either relatively specific or broad detection of MLO DNA in infected host tissues, depending on the primer pair used. To investigate the MLOs associated with grapevine yellows in this study, we employed several different oligonucleotide primer pairs in separate polymerase chain reactions, including a reaction designed for broad amplification of MLO 16S rRNA gene sequences (28). Amplification of 16S rDNA, followed by restriction analyses of the amplified DNA, is a rapid method that has been shown to permit MLO detection (1) and to allow the classification of MLO strains into groups that coincide with clusters delineated earlier on the basis of dot hybridizations (28). This report describes DNA-based detection of MLOs in naturally diseased grapevines in Italy and the eastern United States and the surprising finding that MLOs associated with grapevine yellows from Italy, Germany, France, and the United States could be delineated into three distinct groups: an aster yellows MLO group, an X-disease MLO group, and an elm yellows MLO group.

MATERIALS AND METHODS

Plants and MLOs. In this report, we use the following terminology for grapevine diseases and associated MLOs. The

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term "grapevine yellows" is used in a general sense to refer to grapevine diseases that exhibit symptoms characteristic of so-called plant "yellows" diseases (reviewed in 16). This includes the disease *flavescence dorée,* which was described in France and extensively characterized by Caudwell (8). Also included are grapevine yellows diseases from northern and southern Italy, which, in accordance with recommendations (2), are termed *flavescence dorée* and southern European (Italy) grapevine yellows, respectively.

Leaf samples from two naturally diseased grapevines (*Vitis vinifera* L. 'Chardonnay') exhibiting symptoms of grapevine yellows were collected in Virginia and designated FDVA1 and FDVA2. MLO sample CA7, collected in Emilia-Romagna, Italy, consisted of leaves from a naturally diseased grapevine (*V. vinifera* 'Caveccia'), plant CA7, also exhibiting symptoms of grapevine yellows. Additional samples were taken from periwinkle or from broadbean plants that had been experimentally inoculated from symptomatic grapevines. *Flavescence dorée* MLO strain FDF (FD70) from France was kindly supplied by A. Caudwell (Institut National de la Recherche Agronomique, Dijon, France) in broadbean (*Vicia faba* L.) that had been experimentally inoculated by leafhoppers, *Euscelidius variegatus* (Kirschbaum) (8,14). MLOs associated with grapevine yellows from Italy and Germany were transmitted from naturally infected cultivated grapevines to plants of periwinkle (*Catharanthus roseus* (L.) G. Don) through the use of dodder (*Cuscuta* sp.). Southern Italy grapevine yellows MLO strain FDB, kindly supplied by V. Savino and B. di Terlizzi (Istituto Agronomico Mediterraneo, Bari, Italy), was from the Puglia region; northern Italy *flavescence dorée* MLO strain FDU, kindly supplied by R. Osler and L. Carraro (Università di Udine, Italy), was from the Friuli-Venezia Giulia region; southern Italy grapevine yellows MLO strain FDR was from the Lazio region around Rome; northern Italy grapevine yellows strains CA1, CH1, SAN1, and SAN2 were from the Emilia-Romagna region (13); and grapevine yellows-associated MLO strain FDG from Germany was kindly supplied by M. Maixner (Institut für Pflanzenshutz im Weinbau, Bernkastel-Kues, Germany). Samples of chokecherry (*Prunus virginiana* L.) collected from fields in New York were kindly supplied by W. A. Sinclair (Cornell University, Ithaca, NY). Maryland aster yellows (AY = AY1 = MDAY) MLO, used as a control, was collected from a naturally diseased periwinkle plant in a field in Beltsville, MD. Other known MLOs used as controls were kindly supplied in singly infected plants of periwinkle, unless specified otherwise, by the researchers indicated: tomato big bud (BB) (J. Dale, University of Arkansas, Fayetteville); clover yellow edge (CYE) in ladino clover (*Trifolium repens* L.), Canada X-disease (CX), and clover phyllody (CPh) (L. N. Chiykowski, Plant Research Center, Agriculture Canada, Ottawa, Ontario); western X-disease (WX) (B. C. Kirkpatrick, University of California, Davis); elm yellows (EY) and ash yellows (AshY) (W. A. Sinclair); beet leafhopper-transmitted virescence (VR) (G. N. Oldfield, University of California, Riverside); and potato witches'-broom (PWB) (C. Hiruki, University of Alberta, Edmonton, Alberta, Canada). MLOs in periwinkle were maintined by grafting in a white-flowered clone of periwinkle in the greenhouse. Healthy plants of grapevine were grown from seed in the greenhouse.

PCR analyses. The following seven pairs of oligonucleotide primers were used in PCR: r16F2/r16R2, abbreviated F2/R2 (28); r16F4/r16R1, abbreviated F4/R1 (17); G35p/m (18); AY18p/m and AY19p/m (24,34); R16(V)F1/R1 (I.-M. Lee, *unpublished*); and FDU49f/r (R. E. Davis, J. P. Prince, and E. L. Dally, *unpublished*). The specificities of the oligonucleotide pairs in priming amplification of DNAs from control MLOs were previously determined as follows: oligonucleotide pair F2/R2 primed amplification of a 16S rDNA sequence from all tested known MLOs (28); F4/R1 primed cluster-specific amplification of a 16S rDNA sequence from MLOs in the aster yellows (AY) MLO strain cluster (17); G35p/m primed amplification of a DNA sequence from Italian periwinkle virescence MLO and type II subcluster MLOs in the AY MLO strain cluster (18); AY18p/m primed amplification of a DNA sequence from type II, type III, and some type I MLOs in the AY MLO strain cluster (24); AY19p/m primed amplification of a DNA sequence from all tested type II and some type I subcluster MLOs from the AY MLO strain cluster (24); R16(V)F1/R1 primed cluster-specific amplification of 16S rDNA from elm yellows MLOs that infect elm in United States and Italy (I.-M. Lee, *unpublished*). The specificity of DNA amplification with FDU49f/r is reported here.

Total nucleic acid for use as template in PCR was extracted from healthy and infected plants of periwinkle and broadbean as previously described (25). For extraction of nucleic acid from grapevine, approximately 2 g of fresh leaf veinal tissue from grapevine was frozen in liquid nitrogen and pulverized or stored at −80 C for further use. The pulverized tissue was mixed with 7 ml of filter-sterilized (0.45-μm pore diameter) tissue suspension buffer and stirred with a glass rod for 60 s. Tissue suspension buffer contained 100 mM $K_2HPO_4 \cdot H_2O$, 30 mM KH_2PO_4, 10% sucrose, 0.15% bovine serum albumin Fraction V, 2% polyvinylpyrrolidone-10, and 25 mM L-ascorbic acid. Ascorbic acid was added just before use, and the pH was adjusted to 7.6 with NaOH. The tissue-buffer mixture was filtered through Miracloth (Calbiochem, San Diego, CA) and centrifuged at 20,000 *g* for 20 min. The resultant pellet was resuspended in extraction buffer, and nucleic acid was extracted as described (25). DNA was extracted from batches of five FDF MLO-infected *E. variegatus* by a method described previously for extraction of plant nucleic acid (23). PCR mixtures contained 50 ng of template DNA from healthy or diseased plant tissues (amount estimated from gel electrophoretic comparisons with standards), 0.4 μM of each primer of a pair, 1.5 mM $MgCl_2$, 10 mM Tris-HCl (pH 8.3), 50 mM KCl, 0.001% gelatin, 200 μM each of dATP, dCTP, dTTP, and dGTP, and 1.25 units of *Taq* polymerase (Boehringer-Mannheim, Indianapolis, IN) in a total volume of 50 μl. Reaction mixtures containing sterile, deionized water in place of template DNA served as negative controls. The reaction mixture was overlaid with 25 μl of mineral oil. The PCR was performed in a Perkin-Elmer (Norwalk, CT) thermal cycler. After initial denaturation for 2 min at 94 C, 35 cycles of amplification were carried out as follows: denaturation for 1 min at 94 C, annealing for 2 min at 50 C, and extension for 3 min at 72 C. The final extension step was lengthened by 7 min, and then the reactions were held at 4 C. PCR products were analyzed by electrophoresis in a 1% agarose gel, stained with ethidium bromide, and visualized with a UV transilluminator.

Fig. 1. Symptoms typical of grapevine yellows in naturally infected grapevine (*Vitis vinifera* 'Chardonnay') in Virginia.

Restriction analyses. Products from polymerase chain reactions that contained F2/R2 and G35p/m primer pairs were subjected to analyses by digestion with restriction endonucleases. Aliquots (5 μl) of the reaction mixtures were digested with 15 units of *Alu*I (Sigma Chemical Co., St. Louis, MO), *Mse*I (Sigma), *Kpn*I (Gibco-BRL, Gaithersburg, MD), or *Hpa*II (New England Biolabs, Beverly, MA) according to the manufacturers' instructions. Restriction patterns of digested DNAs were analyzed by electrophoresis in 6% polyacrylamide gels, stained with ethidium bromide, and visualized with a UV transilluminator.

RESULTS

Symptoms in diseased grapevines. Diseased grapevines sampled in Virginia and Italy exhibited symptoms resembling those of *flavescence dorée* and grapevine yellows described in France, Italy, and New York (10,11,32). Symptoms exhibited in grapevine sampled in Virginia included withering and abortion of flower clusters, rolling of lateral leaf margins, overlapping leaf growth, and veinal yellowing and necrosis in leaves (Fig. 1).

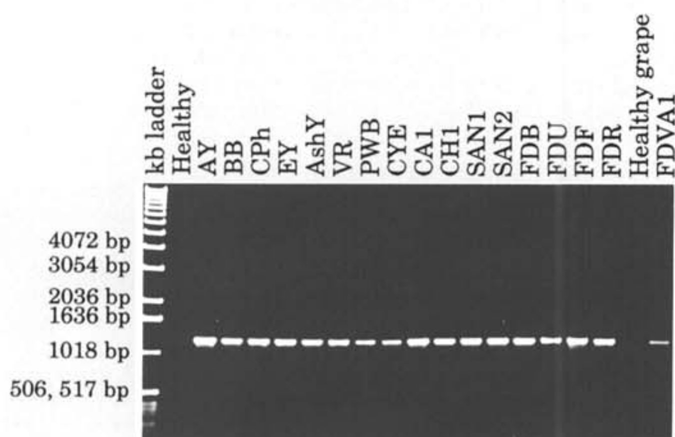

Fig. 2. Detection of mycoplasmalike organisms (MLOs) by polymerase chain reaction amplification of a 16S rRNA gene sequence primed by the oligonucleotide pair r16SF2/R2. Oligonucleotides r16SF2/R2 were designed on the basis of the sequence determined for the 16S rRNA gene from an aster yellows MLO strain (28). Template DNA was extracted from healthy or diseased plants of periwinkle (*Catharanthus roseus*), broadbean (*Vicia faba*), or grapevine (*Vitis vinifera*) infected by MLOs. CA1, CH1, SAN1, SAN2, FDB, FDU, and FDR are MLO strains associated with grapevine yellows from Italy in periwinkle plants; FDF = MLO strain associated with grapevine *flavescence dorée* disease from France in broadbean; FDVA1 = MLO strain associated with grapevine yellows in naturally infected grapevine from Virginia; AY = Maryland aster yellows; BB = tomato big bud; CPh = clover phyllody; EY = elm yellows; AshY = ash yellows; VR = beet leafhopper-transmitted virescence; PWB = potato witches'-broom; CYE = clover yellow edge; healthy = healthy periwinkle.

Detection of MLOs in naturally infected grapevine. When reaction mixtures contained the F2/R2 primer pair and template DNA from either of two symptomatic Chardonnay grapevines cultivated in Virginia or from a symptomatic Caveccia grapevine from Emilia-Romagna, Italy, amplification of a 1.2-kb DNA fragment was observed, which indicated the presence of MLOs (termed strains FDVA1, FDVA2, and CA7, respectively) in the plants. Results from plant sample FDVA1 are shown in Figure 2. Strain FDVA2 was not further analyzed. Strain CA7 data is not shown. No DNA amplification was observed when the template consisted of DNA extracted from healthy grapevine.

Detection of grapevine yellows MLOs in experimentally inoculated plants. MLOs were detected by the amplification of a 1.2-kb 16S rDNA fragment in all reaction mixtures containing oligonucleotide primer pair F2/R2 and template DNA extracted from plants of periwinkle or broadbean singly infected by MLO strains associated with grapevine yellows diseases in France and Italy (Fig. 2). This DNA fragment was the same size as that amplified from control DNA samples from periwinkle plants singly infected by aster yellows, elm yellows, ash yellows, beet leafhopper-transmitted virescence, clover yellow edge, and potato witches'-broom MLOs (Fig. 2). No DNA amplification was observed when the template consisted of control DNA extracted from healthy plants.

Differentiation of grapevine yellows MLOs using PCR. Distinctions among several grapevine yellows MLOs and their affiliations with certain known control MLOs were first indicated in the present work by differential PCR amplification of DNA with various oligonucleotide primer pairs (Table 1). Primer pairs F4/R1 and G35p/m primed amplification of DNA fragments of about 660 and 1,200 bp, respectively, from all of the MLOs associated with grapevine yellows except strains FDU, FDF, and the Virginia grapevine MLO strain FDVA1. As previously reported (17,18), F4/R1 also primed amplification of DNA from aster yellows MLO cluster control strains AY, BB, and CPh; and G35p/m primed amplification of DNA from control MLOs AY and CPh. AY18p/m primed amplification of a DNA fragment of approximately 1,600 bp only from FDB, AY, and CPh, and AY19p/m primed amplification of a DNA fragment of approximately 1,000 bp only from FDR, FDG, and AY (Table 1).

The clear separation of strains FDF, FDU, and FDVA1 from the other grapevine yellows MLOs was confirmed in further experiments. For example, oligonucleotide pair FDU49f/r primed

TABLE 1. Polymerase chain reaction amplifications of DNA from mycoplasmalike organisms (MLOs) associated with grapevine yellows using different oligonucleotide primer pairs

Tissue[a] MLO strain	Geographic origin	F4/R1[b]	G35p/m	AY18p/m	AY19p/m	FDU49f/r	R16(V)F1/R1	F2/R2
Grapevine								
FDVA1	Virginia	−	−	−	−	−	−	+
CA7	Northern Italy (Emilia-Romagna)	+	+	−	−	−	−	+
Periwinkle								
FDU	Northern Italy (Friuli-Venezia-Giulia)	−	−	−	−	+	−	+
FDB	Southern Italy (Puglia)	+	+	+	−	−	−	+
FDR	Central Italy (Lazio)	+	+	−	+	−	−	+
CA1	Northern Italy (Emilia-Romagna)	+	+	−	−	−	−	+
CH1	Northern Italy (Emilia-Romagna)	+	+	−	−	−	−	+
SAN1, SAN2	Northern Italy (Emilia-Romagna)	+	+	−	−	−	−	+
FDG	Germany	+	+	−	+	−	−	+
Broadbean								
FDF	France	−	−	−	−	−	+	+

[a]Template DNA was extracted from MLO-infected tissues of grapevine, periwinkle, or broadbean as indicated. Grapevine tissues were collected from naturally inoculated plants growing in commercial vineyards. Periwinkle and broadbean were experimentally inoculated plants grown in the greenhouse.

[b]MLO-specific amplified DNAs were approximately 660, 1,200, 1,600, 1,000, 2,800, and 1,100 bp when primer pairs F4/R1, G35p/m, AY18p/m, AY19p/m, FDU49f/r, and R16(V)F1/R1 were employed, respectively. − = No MLO-specific DNA amplification; + = MLO-specific DNA amplification. No MLO-specific DNA amplification was observed when the template consisted of DNA from healthy plants.

amplification of a DNA fragment of approximately 2,800 bp from FDU MLO and clover yellow edge (a strain in the X-disease MLO strain cluster) but did not prime amplification of DNA of any other grapevine strains studied, including FDVA1 (Fig. 3). Oligonucleotide pair R16(V)F1/R1 primed amplification of a 1,100-bp DNA fragment only from FDF MLO (Table 1).

Restriction analysis of PCR-amplified MLO DNA sequences. RFLP patterns of amplified 16S ribosomal RNA gene sequences differentiated the MLOs associated with grapevine yellows. Initial results that indicated differences in RFLP patterns (data not shown) were obtained by analysis of 500-bp fragments of 16S rDNA amplified with a primer pair designed in other work (B. D. Mogen, R. E. Davis, and J. A. Foster, *unpublished*). Subsequently, we analyzed the products from F2/R2-primed 16S rDNA amplification to compare RFLP patterns with those of known MLOs studied earlier (28). Representative results from single enzyme digestions with *Alu*I, *Mse*I, and *Kpn*I are depicted in Figures 4 and 5. The 16S rDNAs from MLO strains FDR, FDB, CA1, CH1, SAN1, SAN2, and FDG exhibited identical RFLP patterns, which were indistinguishable from those obtained with 16S rDNA from the aster yellows MLO cluster control strains AY and BB, whether digestion was with *Alu*I or with *Kpn*I. However, after digestion with *Mse*I, only 16S rDNA from strain FDR exhibited the same pattern as did AY and BB. Only MLO strain FDG exhibited the same *Mse*I RFLP pattern as CPh (Figs. 4 and 5). FDB, CA1, CH1, SAN1, and SAN2 differed from AY and BB and from FDR by the presence of an *Mse*I site within a sequence that corresponded to the 300-bp fragment seen in

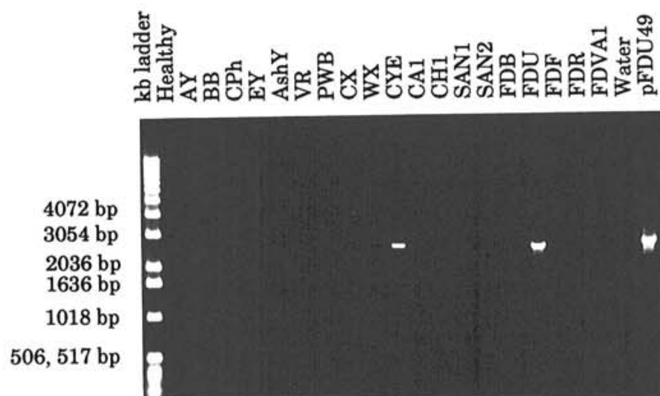

Fig. 3. DNA sequence amplification primed in polymerase chain reactions (PCR) by oligonucleotide pair FDU49f/r. Oligonucleotides FDU49f/r were designed on the basis of partial nucleotide sequence determined for cloned DNA fragment FDU49 from the *flavescence dorée* mycoplasmalike organism (MLO) strain FDU from northern Italy. WX = western X-disease; CX = Canada X-disease; pFDU49 = positive control template DNA of plasmid containing cloned fragment FDU49 of DNA from MLO strain FDU; water = negative PCR control with no DNA and only water as the template. CA1, CH1, SAN1, SAN2, FDB, FDU, and FDR are MLO strains associated with grapevine yellows from Italy in periwinkle plants; FDF = MLO strain associated with grapevine *flavescence dorée* disease from France in broadbean; FDVA1 = MLO strain associated with grapevine yellows in naturally infected grapevine from Virginia; AY = Maryland aster yellows; BB = tomato big bud; CPh = clover phyllody; EY = elm yellows; AshY = ash yellows; VR = beet leafhopper-transmitted virescence; PWB = potato witches'-broom; CYE = clover yellow edge; healthy = healthy periwinkle. MLO strains WX, CX, and CYE are members of the X-disease MLO strain cluster and are included to illustrate that FDU49f/r primes amplification of DNA from FDU and CYE but not from CX or WX.

Fig. 4. Restriction fragment length polymorphism analysis of products from amplification of the mycoplasmalike organism (MLO) 16S rRNA gene sequence primed by oligonucleotide pair r16SF2/R2. Template DNA was extracted from healthy plants or plants infected by MLO strains associated with grapevine yellows or by control MLO strains. Amplified DNA was digested with **A,** *Alu*I, **B,** *Mse*I, or **C,** *Kpn*I. CA1, CH1, SAN1, SAN2, FDB, FDU, and FDR are MLO strains associated with grapevine yellows from Italy in periwinkle plants; FDF = MLO strain associated with grapevine *flavescence dorée* disease from France in broadbean; AY = Maryland aster yellows; BB = tomato big bud; CPh = clover phyllody; EY = elm yellows; AshY = ash yellows; VR = beet leafhopper-transmitted virescence; PWB = potato witches'-broom; CYE = clover yellow edge; healthy = healthy periwinkle.

A
Phi-X174/HaeIII, Healthy, AY, BB, CPh, EY, AshY, VR, PWB, CYE, CA1, CH1, SAN1, SAN2, FDB, FDU, FDF, FDR
872 bp, 603 bp, 310 bp, 271 bp, 234 bp, 194 bp, 118 bp, 72 bp

B
603 bp, 310 bp, 271 bp, 234 bp, 194 bp, 118 bp, 72 bp

C
1353 bp, 1078 bp, 872 bp, 603 bp, 310 bp, 271 bp, 234 bp, 194 bp

amplified 16S rDNA of AY, BB, and CPh.

MLO strain FDU and the Virginia grapevine MLO strain FDVA1 exhibited identical RFLP patterns when their 16S rDNA amplification products were singly digested with all three of the endonucleases (*Mse*I digest is shown in Figure 6). These patterns were the same as those observed for X-disease MLO cluster strains CX and CYE (28). In regions of the northeastern United States where grapevine yellows has been observed (32, 35), X-disease is also found (22). Since chokecherry is known to be a natural source of inoculum for this disease in cultivated stone fruits (33), we examined the possible presence in chokecherry of an MLO related to strain FDVA1. Presence of an MLO in the chokecherry was indicated by amplification of a DNA fragment of about 1,200 bp in PCR with primer pair F2/R2 (data not shown). Amplified 16S rDNA from chokecherry gave the same RFLP pattern after *Mse*I digestion as did 16S rDNA from FDU, CYE, and CX MLOs, and the Virginia grapevine MLO strain FDVA1 (Fig. 6). Thus, it appears that chokecherry also was infected by an MLO belonging to the X-disease MLO strain cluster.

Since *Hpa*II digestion of 16S rDNA was found previously to distinguish subgroups within the X-disease MLO group (28), we compared FDU, FDVA1, and the chokecherry MLO with known control X-disease MLOs by this method. *Hpa*II RFLP analyses delineated two subgroups: one that contained FDVA1, CX, and WX MLOs, and the chokecherry MLO, and the other that contained FDU and CYE MLOs (Fig. 7).

Whereas RFLP analyses of 16S rDNA distinguished FDF from the other grapevine-infecting MLOs, the patterns exhibited by DNA from FDF were identical to those of 16S rDNA from elm yellows MLO (Figs. 4 and 5). The insect vector source (*E. variegatus*) (data not shown) and the plant source of FDF MLO DNA gave the same results. Relatedness between FDF and elm yellows MLOs was also indicated in separate experiments in which probe FD2 (kindly provided by E. Boudon-Padieu, A. Caudwell, and X. Daire) hybridized with DNA of elm yellows MLO but not with DNA of several other MLOs (data not shown), which confirms earlier hybridization data of Daire et al (14).

The RFLP pattern of *Mse*I-digested 16S rDNA amplified from MLO CA7 DNA template exhibited DNA fragments similar in size and number to those observed for DNA amplified from templates derived from control strains in the AY MLO strain cluster, but several additional DNA fragments were observed (data not shown).

The four MLOs (CA1, CH1, SAN1, and SAN2) that originated from grapevines in Emilia-Romagna, Italy, were not differentiated from each other on the basis of DNA amplification with the various primer pairs (Table 1) or by the restriction analysis of 16S rDNA (Fig. 4). Therefore, we analyzed products obtained in PCR with the G35p/m primer pair and DNA templates from plants infected by these MLOs or by strains FDB or FDR. Our rationale was that a randomly cloned fragment of the MLO

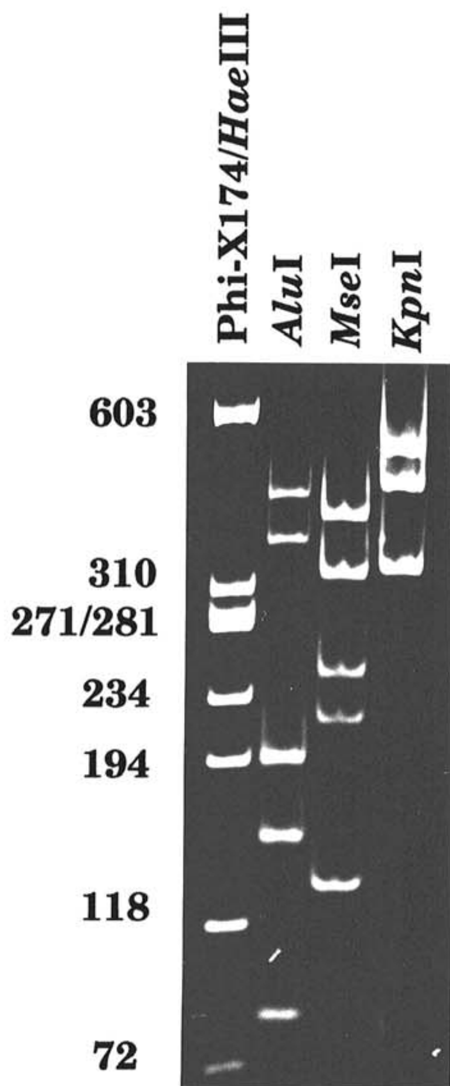

Fig. 5. Restriction fragment length polymorphism analysis of products from amplification of the mycoplasmalike organism (MLO) 16S rRNA gene sequence primed by oligonucleotide pair r16SF2/R2. Template DNA was extracted from periwinkle plants infected by MLO strain FDG. Amplified DNA was digested by *Alu*I, *Mse*I, or *Kpn*I.

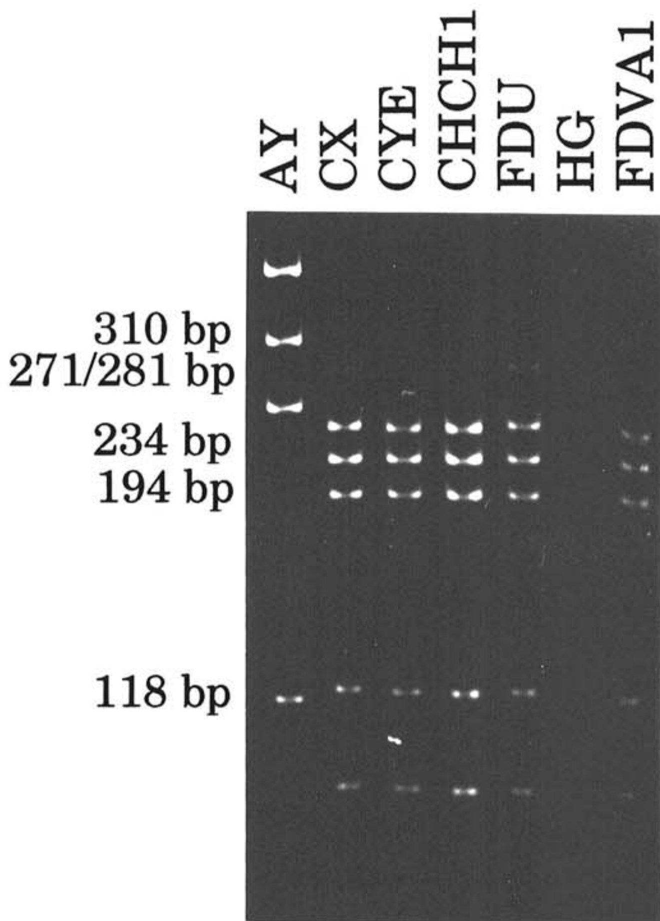

Fig. 6. Restriction fragment length polymorphism analysis of polymerase chain reaction-amplified 16S rDNA comparing Virginia grapevine yellows mycoplasmalike organism (MLO) strain FDVA1, northern Italy grapevine *flavescence dorée* MLO strain FDU, and an MLO (strain CHCH1) detected in field-collected chokecherry from New York State with known strains in the X-disease MLO strain cluster. AY = Maryland aster yellows; CX = Canada X-disease; CYE = clover yellow edge; and HG = healthy grapevine. The oligonucleotide primer pair was r16SF2/R2. Amplified 16S rDNA was digested with *Mse*I.

genome may be more polymorphic than the 16S rDNA sequence. The results distinguished among all six strains with the exception of SAN1 and SAN2, which exhibited identical RFLP patterns in this test (Fig. 8).

DISCUSSION

In this study, an unexpected diversity was revealed among MLOs associated with grapevine yellows. Previous work (15) had indicated that at least two distinct genomic clusters were represented by grapevine-infecting MLOs found in Italy. The present data indicate for the first time that MLOs associated with grapevine yellows in the United States, Italy, Germany, and France represent no less than three distinct groups (Table 2).

This work illustrates molecular detection of MLOs in naturally diseased grapevines cultivated in Virginia and Italy. The symptoms exhibited by the diseased grapevines were similar to those previously described for *flavescence dorée* in France and Italy and for grapevine yellows reported in New York (10,11,32). The data are the first to establish the occurrence of an MLO in association with grapevine yellows in Virginia, and they identify the Virginia MLO strain FDVA1 as a member of the X-disease MLO group.

Interestingly, both the MLO strain FDVA1, associated with grapevine yellows in Virginia, and the *flavescence dorée* MLO strain FDU from northern Italy were classified in the X-disease MLO strain cluster in this work, although the *flavescence dorée* MLO strain FDF from France was not. This finding suggests the possibility that the same or related MLOs might infect both grapevine and stone fruits, causing X-disease in stone fruits and grapevine yellows or *flavescence dorée* in susceptible cultivars of grapevine. Thus, it may be important to investigate whether natural sources of inoculum for grapevine infection may also serve as sources of inoculum for MLO infection in stone fruits and vice versa. However, disease epidemiology could be significantly influenced by differences in genomes among X-disease MLO cluster strains. For example, in work delineating the X-disease MLO strain cluster (27), RFLP patterns of chromosomal DNA indicated that western X-disease and Canada (eastern) X-disease MLOs from stone fruits were more closely related to one another than to a third cluster member, clover yellow edge MLO. Our results from PCR analyses with primer pair FDU49f/r and from *Hpa*II digestion of 16S rDNA indicate that Italy grapevine MLO strain FDU may be more closely related to clover yellow edge MLO than to Canada X- and western X-disease MLOs, whereas the grapevine MLO strain FDVA1 from the United States may be more closely related to Canada X- and western X-disease MLOs than to clover yellow edge MLO (Table 2).

Since strains of MLOs associated with grapevine yellows from both the United States and northern Italy were related to X-disease MLOs, it may be worthwhile to investigate a possible relationship among insect vectors of MLOs associated with grapevine yellows and with X-disease and similar diseases of stone fruits in northern Italy and elsewhere. *S. titanus,* a vector of *flavescence dorée*-grapevine yellows pathogens in France and northern Italy (8,21,36), is native to North America, where it may also vector grapevine yellows MLOs (8,19,30). However, other vectors may also be important in the spread of these diseases

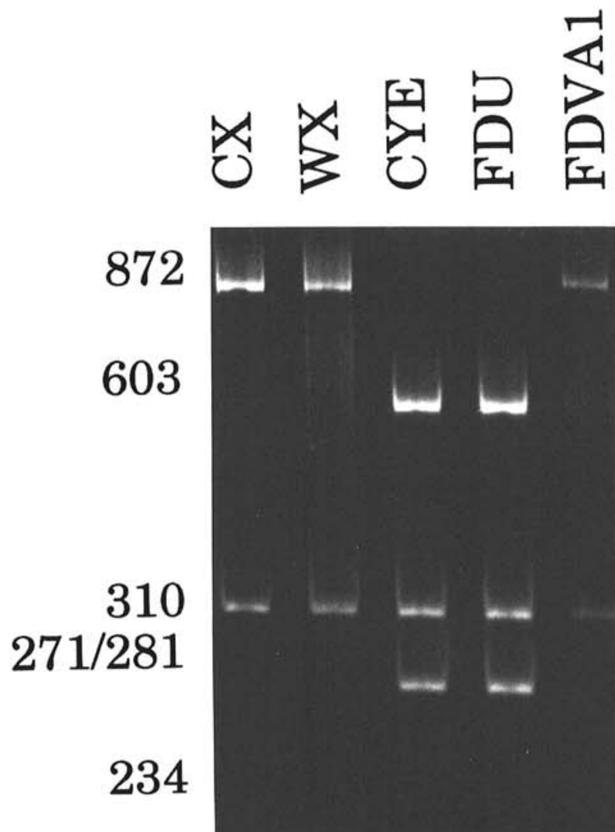

Fig. 7. Restriction fragment length polymorphism analysis of 16S rDNA distinguishing Virginia grapevine yellows strain FDVA1, western X-disease (WX), and Canada X-disease (CX) mycoplasmalike organisms (MLOs) from MLOs associated with clover yellow edge (CYE) and northern Italy grapevine *flavescence dorée* (FDU). Oligonucleotides used to prime amplification of 16S rDNA in polymerase chain reaction analyses were r16SF2/R2. Amplified 16S rDNA was digested with *Hpa*II.

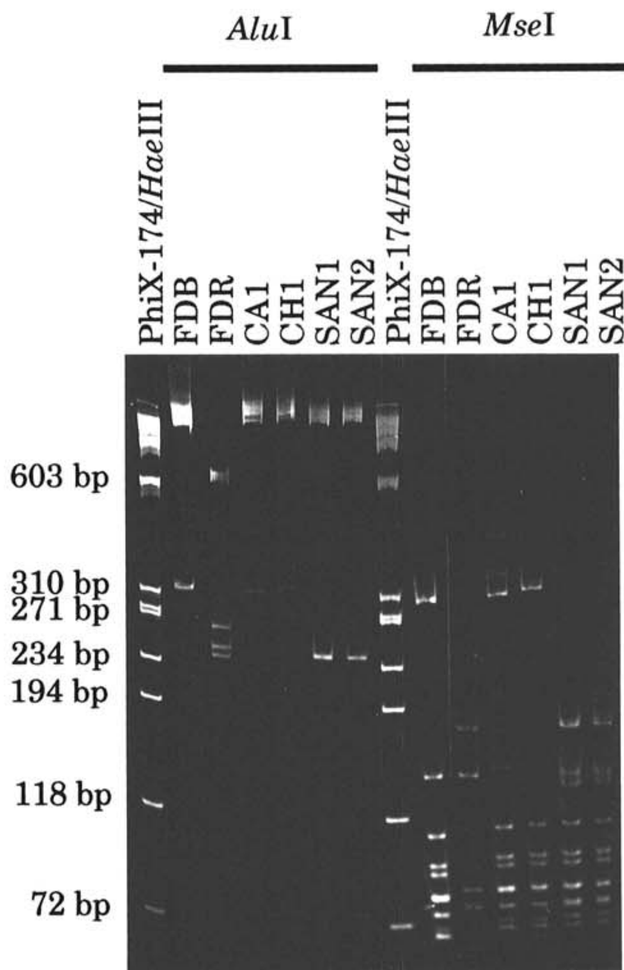

Fig. 8. Restriction fragment length polymorphism analysis of products of DNA amplification primed by oligonucleotide pair G35p/m. Oligonucleotides G35p/m were designed on the basis of partial sequence determined for a cloned fragment of DNA from Italian periwinkle virescence mycoplasmalike organism (MLO) (18). Template DNA was extracted from healthy plants or plants infected by MLO strains associated with grapevine yellows or by control MLO strains. CA1, CH1, SAN1, SAN2, FDB, and FDR are MLO strains associated with grapevine yellows from Italy in periwinkle plants. Amplified DNA was digested with *Alu*I or *Mse*I.

in the United States and Europe. In particular, it seems reasonable to speculate that MLOs that cause grapevine yellows disease might be transmitted by leafhoppers capable of transmitting X-disease MLOs. It would be interesting to determine whether X-disease vectors such as *Fieberiella florii* Stål, for example, which has been found in vineyards in Italy (12), are able to transmit some strains of grapevine yellows MLOs from or to grapevine.

Since the *flavescence dorée* disease described in France is generally regarded as the type example of the disease, it was surprising to find that the *flavescence dorée* MLO strain FDF was distinct from both the FDU MLO from northern Italy and the FDVA1 MLO detected in grapevine in Virginia. FDF was also quite distinct from all other MLOs associated with grapevine yellows in this study; the data indicated a close relationship of FDF to elm yellows MLOs, which have been shown to comprise a distinct genomic strain cluster (26).

Our data on *flavescence dorée* MLO strain FDF are consistent with independent findings (14) in which probe FD2 (IFD2) and six other cloned FDF MLO DNA probes hybridized with DNA of several MLOs, including elm yellows MLO. Elm yellows MLO was the only MLO whose DNA hybridized with all seven of the probes, and one of the probes hybridized with DNA only from elm yellows MLO (14). MLO infection in *flavescence dorée*-diseased grapevine in France was also detected by use of probe FD2 (14). Identification of strain FDF as a member of the elm yellows MLO group may have implications for understanding the epidemiology of *flavescence dorée* disease. Since FDF MLO does appear to be closely related to a strain group including MLOs that infect elm (*Ulmus* spp.), it may be worthwhile to investigate whether strains from elm and grapevine might cross-infect these plant hosts. Although *S. luteolus* Van Duzee is known to transmit elm yellows MLO in North America (3), the natural vector of elm yellows MLO in Europe is not known. In work with FD MLO, *E. variegatus* and *S. titanus* have been used in laboratory transmissions (8,14). It may be useful to determine whether *S. titanus* or *E. variegatus* can transmit elm-infecting MLOs. Further work will be necessary to learn whether grapevine-infecting strains represent a subcluster genomically distinct from strains found in elm.

MLOs originating from grapevine in Germany (MLO strain FDG) and in regions of Italy south of the Po River (MLO strains FDB, FDR, CA1, CH1, SAN1, and SAN2) resembled each other in the PCR amplification of template sequences when oligonucleotide primer pairs F4/R1 and G35p/m were used. Such DNA amplification is characteristic of MLOs in the AY MLO strain cluster and has not been demonstrated in any MLOs that are not members of this cluster (17,18). Whereas all seven of these strains also resembled AY MLO cluster strains in RFLP patterns of *Alu*I- and *Kpn*I-digested 16S rDNA, only strains FDR and FDG exhibited *Mse*I restriction patterns previously observed for any AY MLO cluster strains (28). On the basis of the data, we tentatively classify all seven strains in the AY MLO strain cluster, recognizing that strains FDB, CA1, CH1, SAN1, and SAN2 differ from strains FDR, FDG, and other strains in the cluster. In analogy with the AY MLO cluster strain CPh, which represents a separate subcluster (type) and whose DNA also exhibits an *Mse*I restriction pattern unique among control MLOs used (28), it is possible that the five similar grapevine yellows MLOs from the Emilia-Romagna and Puglia regions of Italy represent a separate and new subcluster within the AY MLO strain cluster. FDR and FDG resemble the Maryland AY and CPh subclusters, respectively (Table 2).

TABLE 2. Classification of mycoplasmalike organisms (MLOs) associated with grapevine yellows in Europe and North America

MLO strain	MLO group classification	Most closely related control strain (s)
FDVA1	X-disease	CX, WX
FDU	X-disease	CYE
FDB	Aster yellows	ND[a]
FDR	Aster yellows	AY
CA1	Aster yellows	ND
CH1	Aster yellows	ND
SAN1, SAN2[b]	Aster yellows	ND
FDG	Aster yellows	CPh
FDF	Elm yellows	EY
CA7	ND	ND

[a]Not determined.

[b]MLO strains SAN1 and SAN2 were not distinguished on the basis of data in this report.

The classification of grapevine MLO strains FDB, CA1, CH1, SAN1, SAN2, FDR, and FDG in the AY MLO strain cluster may have implications for the identification of insect vectors of these pathogens. For example, *S. titanus* has not been reported in southern Italy, and the vector responsible for the spread of grapevine yellows (southern European grapevine yellows) in this region is unknown (31). It may be worthwhile to investigate vector species known to transmit AY MLOs for their ability to transmit certain grapevine-infecting MLOs.

MLO strain CA7, detected in naturally diseased grapevine in the Emilia-Romagna region of Italy, was interesting in that it differed from other MLOs isolated from grapevines in the same region. For example, it resembled strains CA1, CH1, SAN1, and SAN2 in the amplification of AY MLO strain cluster-specific DNA sequences when PCR mixtures contained primer pair F4/R1 or G35p/m. However, this MLO could not be definitively classified, because its 16S rDNA exhibited an RFLP pattern not clearly similar to that characteristic of any one MLO group (data not shown). Instead, the pattern may represent the presence of two different MLOs in the affected vine sampled, one related to AY MLOs and the other not yet determined. This hypothesis is supported by the fact that the observed RFLP DNA fragments from digestion with *Mse*I together represent a DNA sequence of about 2,600 bp. This size is 1,400 bp larger than the size expected based on the positions of primer annealing to target 16S rDNA. The identity(ies) of the MLO(s) referred to as CA7 is under further study.

Previously, we reported molecular evidence from dot hybridizations and RFLP analyses of chromosomal DNA indicating that grapevine yellows in Italy was associated with at least two distinct MLOs represented by strains FDU from northern Italy and FDB from southern Italy (15). Results from the present work are completely consistent with this conclusion. Our data indicate that most MLOs associated with grapevine yellows investigated here, with the exception of strains FDU, FDVA1, and FDF, are members of the AY MLO group, that strains FDVA1 and FDU are members of the X-disease MLO group, and that strain FDF belongs to the elm yellows MLO group. Thus far, our data have identified a strain related to X-disease MLOs in grapevine in North America; the data also indicate that at least three distinct MLOs occur in yellows-diseased grapevines in Europe. The breadth of genomic diversity we have observed among grapevine-infecting MLOs was unexpected. Further work will be required to determine the geographic distribution and relative frequencies of grapevine infection by these diverse MLOs and to establish what etiological roles they may have in grapevine *flavescence dorée*-grapevine yellows disease syndromes.

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. Anonymous. 1991. Int. Counc. Study Viruses (and) Virus Dis. Grapevine (ICVG). Newsl. 11, Dec., pp. 1-7.
3. Baker, W. L. 1948. Transmission by leafhoppers of the virus causing phloem necrosis of American elm. Science 108:307-308.
4. Belli, G., Fortusini, A., and Osler, R. 1978. Present knowledge of diseases of the type "flavescence dorée" in vineyards of northern Italy. Monograf. INIA (Inst. Nac. Invest. Agrar.) 18:7-13.
5. Belli, G., Fortusini, A., and Rui, D. 1985. Recent spread of flavescence dorée and its vector in vineyards of northern Italy. Phytopathol.

Mediterr. 24:189-191.

6. Borgo, M., Egger, E., and Corino, L. 1987. Presenza e diffusione in Italia della "flavescenza dorata" malattia responsabile di gravi deperimenti su vitigni europei. Pages 209-236 in: Atti del Convegno sulla flavescenza dorata della vite. Fondazione "Sergio Bolla," Verona, Italy.
7. Boubals, D. 1987. Reflets negatifs de la flavescence dorée sur le futur de la viticulture européenne. Pages 249-256 in: Atti del Convegno sulla flavescenza dorata della vite. Fondazione "Sergio Bolla," Verona, Italy.
8. Caudwell, A. 1990. Epidemiology and characterization of Flavescence dorée (FD) and other grapevine yellows. Agronomie 10:655-663.
9. Caudwell, A., Larrue, J., Kuszala, C., and Bachelier, J. C. 1971. Pluralité des jaunisses de la vigne. Ann. Phytopathol. 3:95-105.
10. Caudwell, A., and Schvester, D. 1970. Flavescence dorée. Pages 201-207 in: Virus Diseases of Small Fruits and Grapevines: A Handbook. N. W. Frazier, ed. University of California, Berkeley.
11. Credi, R., and Babini, A. R. 1984. Casi epidimici di Giallume della vite in Emilia-Romagna. Vignevini 1:35-39.
12. Credi, R., Babini, A. R., and Petrini, C. 1987. Ulteriori osservazioni su una malattia della vite simile alla flavescenza dorata in Emilia-Romagna. Pages 141-148 in: Atti del Convegno sulla flavescenza dorata della vite. Fondazione "Sergio Bolla," Verona, Italy.
13. Credi, R., and Santucci, A. 1992. Dodder transmission of mycoplasmalike organisms (MLOs) from grapevines affected by a flavescence dorée-type disease to periwinkle. Phytopathol. Mediterr. 31:154-162.
14. Daire, X., Boudon-Padieu, E., Berville, A., Schneider, B., and Caudwell, A. 1992. Cloned DNA probes for detection of grapevine Flavescence dorée mycoplasmalike organism (MLO). Ann. Appl. Biol. 121:95-103.
15. Davis, R. E., Dally, E. L., Bertaccini, A., Credi, R., Osler, R., Savino, V., Carraro, L., di Terlizzi, B., Lee, I.-M., and Barba, M. 1993. Restriction fragment length polymorphism analyses and dot hybridizations distinguish mycoplasmalike organisms associated with *flavescence dorée* and southern European grapevine yellows disease in Italy. Phytopathology 83:772-776.
16. Davis, R. E., and Lee, I.-M. 1982. Pathogenicity of spiroplasmas, mycoplasmalike organisms, and vascular-limited fastidious walled bacteria. Pages 491-513 in: Phytopathogenic Bacteria, vol. 1. M. Mount and G. Lacy, eds. Academic Press, New York.
17. Davis, R. E., and Lee, I.-M. 1993. Cluster-specific polymerase chain reaction amplification of 16S rDNA sequences for detection and identification of mycoplasmalike organisms. Phytopathology 83:1008-1011.
18. 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.
19. De Long, D. M. 1939. A review of the genus *Scaphoideus*. Proc. Entomol. Soc. Wash. 41:33-45.
20. Deng, S., and Hiruki, C. 1991. Amplification of 16S rRNA genes from culturable and non-culturable mollicutes. J. Microbiol. Methods 14:53-61.
21. Fortusini, A., Saracchi, M., and Belli, G. 1989. Transmission of grapevine Flavescence dorée by *Scaphoideus titanus* Ball in Italy. Vignevini 9:43-46.
22. Gilmer, R. M., Palmer, D. H., Schaefers, G. A., and McEwen, F. L. 1966. Leafhopper transmission of X-disease of stone fruits in New York. Page 22 in: N.Y. State Agric. Exp. Stn. Bull. 813.
23. 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.
24. 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.
25. 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.
26. Lee, I.-M, Davis, R. E., Sinclair, W. A., Dewitt, N. D., and Conti, M. 1993. Genetic relatedness of mycoplasmalike organisms detected in *Ulmus* spp. in the United States and Italy by means of DNA probes and polymerase chain reactions. Phytopathology 83:829-833.
27. 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.
28. Lee, I.-M., Hammond, R. W., Davis, R. E., and Gundersen, D. E. 1993. Universal amplification and analysis of pathogen 16S rDNA for classification and identification of mycoplasmalike organisms. Phytopathology 83:834-842.
29. Lherminier, J., Terwisscha van Scheltinga, T., Boudon-Padieu, E., and Caudwell, A. 1989. Rapid immunofluorescent detection of the grapevine flavescence dorée mycoplasmalike organism in the salivary glands of the leafhopper *Euscelidius variegatus* Kbm. J. Phytopathol. 125:353-360.
30. Maixner, M., and Pearson, R. C. 1991. Studies on *Scaphoideus titanus* Ball, a possible vector of grapevine yellows in New York. Pages 193-201 in: Proc. Meet. Int. Counc. Study Viruses (and) Virus Dis. Grapevine (ICVG), 10th. I. C. Rumbos, R. Bovey, D. Gonsalves, W. B. Hewitt, and G. P. Martelli, eds. ICVG, Volos, Greece.
31. Martelli, G. P. 1986. Grapevine diseases induced by phloem- or xylem-limited prokaryotes in Europe, with special reference to Italy. Pages 35-43 in: Fastidious Prokaryotes: Cultivation, Detection, and Associated Economic Problems. R. A. Cappellini and J. M. Wells, eds. Rutgers University Press, New Brunswick, NJ.
32. Pearson, R. C., Pool, R. M., Gonsalves, D., and Goffinet, M. C. 1985. Occurrence of flavescence dorée-like symptoms on "White Riesling" grapevines in New York, USA. Phytopathol. Mediterr. 24:82-87.
33. Rosenberger, D. A., and Jones, A. L. 1978. Leafhopper vectors of the peach X-disease pathogen and its seasonal transmission from chokecherry. Phytopathology 68:782-790.
34. Schaff, D., Lee, I.-M., and Davis, R. E. 1992. Sensitive detection and identification of mycoplasmalike organisms in plants by polymerase chain reactions. Biochem. Biophys. Res. Comm. 186:1503-1509.
35. Uyemoto, J. K. 1974. A new disease affecting the grapevine variety De Chaunac. (Abstr.) Proc. Am. Phytopathol. Soc. 1:146.
36. Vidano, C. 1964. Scoperta in Italia della *Scaphoideus littoralis* Ball cicalina americana collegata alla "Flavescence dorée" della vite. L'Italia Agric. 101:1031-1049.