Restriction Fragment Length Polymorphism Analyses and Dot Hybridizations Distinguish Mycoplasmalike Organisms Associated with *Flavescence Dorée* and Southern European Grapevine Yellows Disease in Italy

Robert E. Davis, Ellen L. Dally, Assunta Bertaccini, Ing-Ming Lee, Rino Credi, Ruggero Osler, Vito Savino, Luigi Carraro, Biagio Di Terlizzi, and Marina Barba

First, second, and fourth authors, Molecular Plant Pathology Laboratory, Agricultural Research Service, USDA, Beltsville, MD; third and fifth authors, Universita di Bologna, Bologna, Italy; sixth and eighth authors, Universita di Udine, Udine, Italy; seventh and ninth authors, Istituto Agronomico Mediterraneo, Bari, Italy; tenth author, Istituto Sperimentale per la Patologia Vegetale, Rome, Italy.

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ABSTRACT

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Biotinylated cloned DNA probes were employed in dot hybridizations and restriction fragment length polymorphism (RFLP) analyses to compare mycoplasmalike organisms (MLOs) associated with two grapevine yellows diseases (strain FDU of *flavescence dorée* MLO from northern Italy and strain FDB of southern European grapevine yellows MLO from southern Italy) and Italian periwinkle virescence disease (MLO strain G from northern Italy). Results from dot hybridizations using six probes containing cloned DNA of MLO strain FDU, of MLO strain G, or of American aster yellows MLO strain AY1, revealed that FDU and FDB shared some regions of DNA sequence homology with one another as well as with MLO strains G and AY1, but all four MLOs were mutually distinguished. RFLP patterns of chromosomal DNA from southern European grapevine yellows MLO strain FDB exhibited some similarities with those from MLO strain G but were markedly different from those exhibited by DNA from *flavescence dorée* MLO strain FDU. The results indicate that at least two distinct MLOs are associated with grapevine yellows in Italy.

Flavescence dorée (FD) is among the most serious diseases known in grapevine (*Vitis vinifera* L.). The causal pathogen is believed to be an uncultured mycoplasmalike organism (MLO) that is transmitted to grapevine in the field by the leafhopper *Scaphoideus titanus* Ball (5–7,31). Since the original report of FD disease in France (5), grapevine yellows diseases exhibiting symptoms similar to those characterizing FD have been noted in several countries including Italy, Germany, Romania, Switzerland, Australia, and the United States (3–8,13,18,20–22, 24–31). In some cases, MLOs have been shown to be associated with grapevine yellows diseases, whereas in others the association of MLOs with disease has been inferred on the basis of symptomatology in diseased grapevine. Although the various grapevine yellows diseases closely resemble FD in symptomatology, there is little direct evidence to indicate whether these diseases are forms of FD or whether the pathogen(s) involved may be distinct from the FD agent and/or from one another.

At least two separate grapevine yellows diseases may occur in Europe. In France, two grapevine yellows diseases, termed FD and *bois noir* (BN), elicit very similar or identical symptoms in grapevine and are distinguished on the basis of transmission of the FD pathogen by *S. titanus* and the apparent inability of this insect to transmit the BN disease pathogen (6,7). In Italy, grapevine yellows diseases have been reported to occur in northern and southern regions, but *S. titanus* has been reported to occur only in northern and not in southern Italy (3,8,13,20,21,24,31). Although an insect vector is postulated to be responsible for the spread of grapevine yellows in southern Italy, no vectors have been identified to date. Recently, grapevine yellows occurring in northern Italy has been termed *flavescence dorée* (FD), while that in southern Italy has been termed southern European grapevine yellows (1). Very similar or identical symptoms are exhibited by yellows diseased grapevines growing in both regions of Italy, and these symptoms closely resemble those characteristic of FD and BN in France. Yet the question of a possible relationship between the two grapevine yellows diseases in northern and southern Italy has remained unanswered, and it is not known whether the pathogen in either region may be related to the pathogens causing grapevine yellows diseases elsewhere.

In the present work, we have used cloned MLO DNA probes in dot hybridizations and analyses of chromosomal DNA polymorphisms to compare a strain of FD MLO from northern Italy and a strain of southern European grapevine yellows MLO from southern Italy with one another and with a strain of Italian periwinkle virescence (IPVR) MLO. Comparison of grapevine yellows-associated MLOs with IPVR MLO was prompted by the recovery of the IPVR MLO in seedlings of *Catharanthus roseus* (L.) G. Don that had been placed in a northern Italy vineyard severely affected by FD disease. Our results indicate that at least two genetically related but distinct MLOs are associated with grapevine yellows diseases in Italy (10).

MATERIALS AND METHODS

Plant samples and MLO strains. *Flavescence dorée* (FD) MLO strain FDU (northern Italy grapevine yellows: GYU) was transmitted by means of dodder (*Cuscuta* sp.) to plants of periwinkle (*C. roseus*) from a naturally infected field-grown grapevine (*Vitis vinifera* L. 'Chardonnay') exhibiting symptoms of FD disease in the Friuli-Venezia Giulia region of northern Italy. Southern European grapevine yellows MLO strain FDB was transmitted to *C. roseus* by means of dodder from a naturally infected grapevine (*V. vinifera* 'Susumaniello') exhibiting symptoms of southern European grapevine yellows disease in the Puglia region of southern Italy. Grapevine source plants and recipient periwinkle seedling plants were protected from insect vectors during the transmission trials. IPVR MLO was originally obtained from a

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naturally infected plant of *C. roseus* that had been placed as a seedling trap plant in a northern Italy vineyard that was seriously affected by FD disease. Aster yellows (AY1) was originally collected in a naturally infected field-grown periwinkle plant in Beltsville, MD. The MLOs were maintained separately by grafting in a white-flowered clone of *C. roseus* maintained in an insect-proof greenhouse.

Cloned MLO DNA probes. Cloned MLO DNA probes were developed in previous work by purification of DNA from sieve cells isolated from MLO-infected periwinkle, digestion of DNA with *Eco*RI and *Hin*dIII, and ligation of DNA fragments into plasmid vectors followed by cloning in *Escherichia coli* hosts. Recombinant plasmids, or the cloned DNA inserts in such plasmids, that hybridized with nucleic acid from MLO-infected plants but not with DNA from healthy plants were labeled with biotin-7-dATP (Bethesda Research Laboratories, Gaithersburg, MD) and used as probes. Thus, probes consisted either of recombinant plasmids (p), composed of plasmid vector pSP64 (Promega Biotech, Madison, WI) plus cloned MLO DNA fragment, or of cloned MLO DNA fragments (insert = I) alone. Probes pFDU49 (3.0 kbp) and pFDU59 (1.0 kbp) were biotinylated recombinant plasmids, each containing a fragment of chromosomal DNA from MLO strain FDU (R. E. Davis and E. L. Dally, *unpublished*). Probes G20AI (1.4 kbp), G35I (1.4 kbp), and G39I (4.1 kbp) consisted of biotinylated cloned chromosomal DNA fragments from Italian periwinkle virescence MLO strain G (9); inserts were excised from vector pIBI30 (IBI, New Haven, CT), to which they had been ligated for cloning, because vector sequences hybridized with nucleic acid extracted from healthy plant tissues. Probes pAY18 (1.6 kbp) and pAY22 (2.9 kbp) were biotinylated recombinant plasmids containing cloned fragments of chromosomal DNA from American aster yellows MLO strain AY1 (15).

Dot hybridizations. Plant samples (leaf and/or stem) consisting of 0.3 g of fresh or frozen (−20 C) tissues were submerged in liquid nitrogen and pulverized in a mortar and pestle. Nucleic acid was extracted and denatured and neutralized as previously described (15). Denatured, neutralized samples were applied to nitrocellulose membranes (Schleicher & Schuell, Keene, NH) (about 7 μg of total nucleic acid per spot) as previously described (15). The membranes were then air dried and baked at 80 C for 2 h under vacuum.

Prehybridization, hybridization, washes, membrane blocking, and visualization of hybridized biotinylated probe were performed, with minor modification, according to the procedures of the BluGENE Nonradioactive Research Nucleic Acid Detection System (Bethesda Research Laboratories, Life Technologies, Gaithersburg, MD). Hybridization temperatures were varied to achieve two levels of stringency. Following a prehybridization treatment of 4-h duration, dot hybridizations were carried out for 16 h, either at 42 C (moderate stringency) or at 52 C (high stringency), in the presence of 5× SSC, 1× Denhardt's solution, 5% dextran sulfate, 45% formamide, 20 mM of sodium phosphate (pH 6.5), 0.2 mg of denatured salmon sperm DNA per milliliter, and 0.2 μg of biotin-labeled probe DNA per milliliter. Membranes were then washed twice for 3 min at room temperature in 2× SSC containing 0.1% SDS, twice in 0.2× SSC containing 0.1% SDS, and then twice at 50 C for 15 min in 0.16× SSC containing 0.1% SDS. Membrane blocking and visualization of hybridized biotinylated probe using streptavidin-alkaline phosphatase conjugate (BluGENE Nonradioactive Nucleic Acid Detection System) and nitroblue tetrazolium plus 5-bromo-4-chloro-3-indolyl phosphate, were as previously described (15). Color development was observed briefly under low light at 10-min intervals for up to 1 h. After color had developed sufficiently to reveal positive nucleic acid hybridizations, the membranes were washed for 5 min in a reaction termination solution consisting of 20 mM Tris (pH 7.5) plus 0.5 mM EDTA. In the present work, sensitivity of detection was 1–2 pg of homologous DNA.

RFLP analyses. DNA for RFLP analyses was prepared from 1.0 g of tissue (leaf and/or stem) from a MLO-infected or healthy plant of *C. roseus* by a procedure previously described (2) as modified by Lee et al (17). Tissue was pulverized in liquid nitrogen and the pulverized sample triturated in 2–4 ml of DNA extraction buffer (100 mM Tris-HCl [pH 8.0], 100 mM EDTA, 250 mM NaCl, 100 μg of proteinase K per milliliter). Sarkosyl was added to each sample after trituration to give a final concentration of 1%. The sample was then incubated for 1 h at 55 C and clarified by centrifugation at 4,300 *g* for 10 min. Nucleic acid was precipitated from the supernatant liquid by adding 0.6 volume of isopropanol followed by centrifugation at 7,000 *g* for 15 min. The pellet was resuspended in 1 ml of TE buffer (10 mM Tris-HCl, 1 mM EDTA, pH 8.0) containing 100 μg of proteinase K per milliliter and 0.5% sodium dodecyl sulfate (SDS) and incubated for 1 h at 37 C. The sample was then mixed thoroughly with 175 μl of 5 M NaCl followed by the addition of 140 μl of 10% cetyltrimethylammonium bromide (CTAB) in 0.7 M NaCl. After incubation for 10 min at 65 C, the sample was extracted with an equal volume of chloroform-isoamyl alcohol (24:1), repeatedly until no precipitate was visible at the interface. The aqueous phase was then extracted once with an equal volume of TE-saturated phenol-chloroform-isoamyl alcohol (25:24:1). The nucleic acid was precipitated from the aqueous phase as before. The pellet was then washed with 70% ethanol to remove residual CTAB, briefly allowed to dry, and resuspended in TE buffer.

Nucleic acid samples (8–10 μg of total nucleic acid per sample) from healthy or MLO-infected periwinkle plants were singly digested with *Eco*RI, *Hin*dIII, or *Cla*I or doubly digested with *Eco*RI and *Hin*dIII, at 37 C for 24 h. Complete DNA digestion after 24 h was indicated by observation of identical RFLP patterns from samples digested for 4, 24, or 48 h. Digested samples were electrophoresed in a 0.7% agarose gel, alkali denatured, and transferred to a nitrocellulose membrane by the method of Southern as described by Maniatis et al (19). The membranes were air dried, baked in vacuo, prehybridized, and hybridized with biotin-labeled cloned DNA probes under conditions of moderate (42 C) or high (50 C) stringency as described for dot hybridizations. Two cloned DNA probes, G35I and G39I, each containing a fragment of DNA from IPVR MLO strain G, were

TABLE 1. Comparison of mycoplasmalike organisms (MLOs) associated with grapevine *flavescence dorée* (FDU), southern European grapevine yellows (FDB), Italian periwinkle virescence (G), and American aster yellows (AY1) by dot hybridizations with cloned MLO DNA probes

Probe DNA[a]	Hybr. temp. (C)[b]	Hybridization with nucleic acid sample[c]				
		H	G	FDU	FDB	AY1
pFDU49	42	−[d]	+	+	+	+
	52	−	+	+	+	W
pFDU59	42	−	+	+	+	W
	52	−	W	+	W	−
G20AI	42	−	+	+	+	+
	52	−	+	+	+	−
G39I	42	−	+	+	+	+
	52	−	+	+	+	+
pAY18	42	−	+	+	+	+
	52	−	+	−	+	+
pAY22	42	−	+	+	+	+
	52	−	+	+	−	+

[a]Probes pFDU49, and pFDU59 were biotinylated recombinant plasmids consisting of plasmid vector pSP64 containing cloned DNA fragments from MLO strain FDU. Probes G20AI and G39I consisted of biotinylated cloned MLO strain G DNA fragments (inserts) excised from recombinant plasmids. Probes pAY18 and pAY22 consisted of recombinant plasmids containing cloned DNA fragments from American aster yellows MLO strain AY1.

[b]Hybr. temp., temperature of hybridization reaction.

[c]Nucleic acid was extracted from healthy (H) or MLO-infected plants of *Catharanthus roseus*. G, strain G of Italian periwinkle virescence MLO. FDU, *flavescence dorée* MLO from northern Italy. FDB, southern European grapevine yellows MLO. AY1, American aster yellows MLO strain AY1.

[d]+, positive hybridization signal. W, weak hybridization signal. −, no hybridization signal.

used for RFLP analyses. For each probe/restriction enzyme combination used, the similarity coefficient (F) of strains x and y was calculated according to the relation, $F = 2\ N_{xy}/(N_x + N_y)$, where N_x and N_y are the total numbers of hybridized restriction fragments in strains x and y, respectively, and N_{xy} is the number of hybridized DNA fragments shared by the two strains (23). F values are the means from all probe/restriction enzyme combinations used for RFLP analyses.

RESULTS

Differentiation of MLO strains by dot hybridizations. Results from dot hybridizations of six biotinylated cloned DNA probes against nucleic acid samples from healthy or MLO-infected plants of *C. roseus* are summarized in Table 1. None of the probes hybridized with nucleic acid samples extracted from healthy plants. All nucleic acid samples derived from MLO-infected plants hybridized with several of the probes, but each MLO exhibited a unique pattern of hybridization. When hybridization reactions were carried out under conditions of moderate stringency, nucleic acid samples extracted from plants infected by MLO strains FDU, FDB, G, and AY1 hybridized with all six probes. When hybridization reactions were carried out under conditions of high stringency, nucleic acid samples from plants infected by strain FDU MLO hybridized with probes pFDU49, pFDU59, G20AI, G39I, and pAY22, but did not hybridize with probe pAY18. Under conditions of high stringency, nucleic acid samples from plants infected by strain FDB MLO hybridized with probes pFDU49, pFDU59, G20AI, G39I, and pAY18, but did not hybridize with probe pAY22. Nucleic acid from plants infected by MLO strain AY1 hybridized with probes pFDU49, G39I, pAY18, and pAY22, but did not hybridize with probes pFDU59 and G20AI. All six probes hybridized under conditions of high stringency with nucleic acid from plants infected by MLO strain G.

RFLP analyses. RFLP analyses compared DNA from three MLO strains digested with *Eco*RI, *Hin*dIII, *Cla*I, or *Eco*RI plus *Hin*dIII and probed with cloned DNA G35I or G39I. The DNA was extracted from healthy plants and from plants singly infected by *flavescence dorée* MLO strain FDU, southern European grapevine yellows MLO strain FDB, or strain G of Italian periwinkle virescence MLO. RFLP analysis of *Eco*RI or *Hin*dIII digests probed with G39I is shown in Figure 1. No hybridization of the probe with DNA extracted from healthy plants was observed, but the probe did hybridize with all samples of DNA extracted from MLO-infected plants, consistent with results from dot hybridizations. The RFLP patterns observed for a given MLO DNA/restriction enzyme combination were basically the same at both levels of stringency, but patterns of hybridized DNA bands differed among the three MLOs at both levels of stringency of the hybridization reaction. The RFLP patterns observed for MLO strains FDB and G exhibited similarities to one another in some cases, but both differed markedly from those observed for MLO strain FDU.

RFLP patterns observed following high-stringency Southern hybridizations of restriction endonuclease-digested DNA against probe G35I are shown in Figure 2. When Southern hybridizations were carried out with DNA digested with *Hin*dIII, each exhibited a RFLP pattern distinct from that of the others. None of the bands observed from any one of the three MLOs represented a DNA fragment size in common with either of the other two MLOs. In hybridizations of probe G35I against DNA samples digested with *Eco*RI, each MLO also exhibited an individually distinct RFLP pattern, but strains G and FDB shared in common a band corresponding to a DNA fragment of about 1,400 bp. A corresponding DNA fragment was not detected in restriction digests of DNA from strain FDU. DNA fragments from strain FDU, corresponding to about 4,500 and 5,800 bp, were observed in *Hin*dIII and *Eco*RI digests, respectively, when hybridized with probe G35I.

DNA from MLO strains FDU, FDB, and G which had been doubly digested with *Eco*RI and *Hin*dIII was also probed with G35I (Fig. 3). Again, FDU, FDB, and G MLOs each exhibited individually distinct patterns of DNA fragments hybridizing with probe G35I. In digests from both FDB and G, the probe hybridized with a DNA fragment of approximately 1,400 bp, the size of the MLO DNA probe itself. Detection of a 1,400-bp DNA fragment in *Eco*RI (see Figs. 1 and 2) or *Eco*RI plus *Hin*dIII-digested DNA from strains FDB and G, when probed with G39I and G35I, may be explained by the apparent presence in 4,100-bp probe G39I of a region that is homologous with the 1,400-bp probe G35I. Probe G35I detected a distinctive DNA fragment of about 3,300 bp in the *Eco*RI/*Hin*dIII-digested DNA of FDU MLO.

DNAs from strains FDU, FDB, and G were digested using *Cla*I and probed with G35I and G39I (Fig. 4). Although several

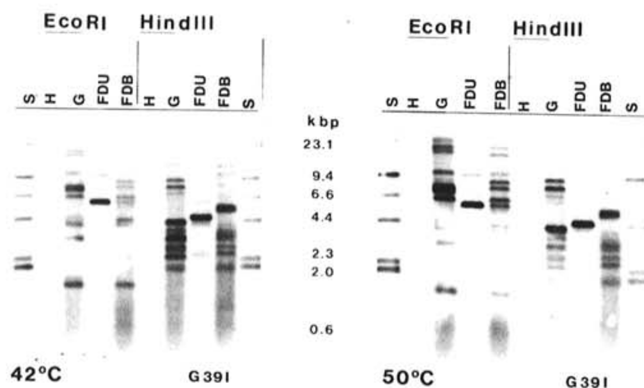

Fig. 1. RFLP analysis of chromosomal DNA of mycoplasmalike organism (MLO) strains associated with *flavescence dorée* (strain FDU), southern European grapevine yellows disease (strain FDB), or Italian periwinkle virescence (strain G). DNA extracted from healthy (H) or MLO-infected plants was singly digested with restriction endonuclease *Eco*RI or *Hin*dIII. Hybridizations were conducted at 42 C (left) or 50 C (right) using biotinylated cloned DNA probe G39I. Lane S contained biotinylated lambda DNA/*Hin*dIII fragments as markers.

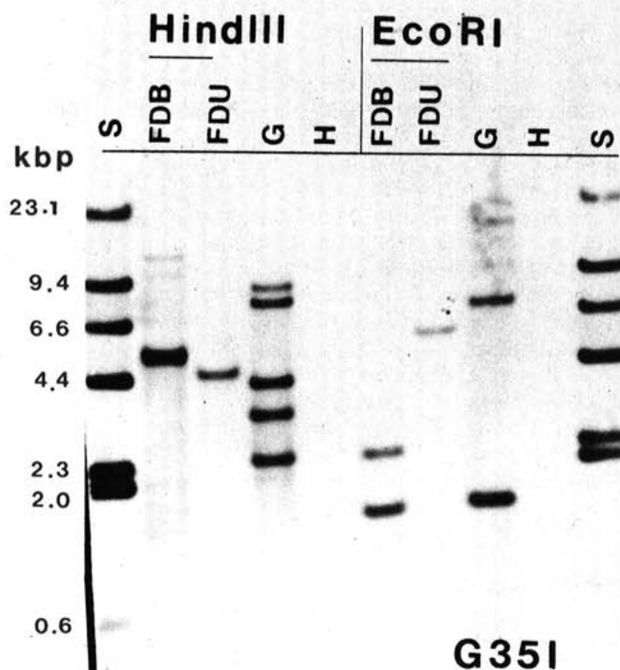

Fig. 2. RFLP analysis of chromosomal DNA of MLO strains associated with *flavescence dorée* (FDU), southern European grapevine yellows disease (FDB), or Italian periwinkle virescence (G). DNA from healthy or MLO-infected plants was singly digested with restriction endonuclease *Eco*RI or *Hin*dIII. Hybridizations were conducted at 50 C using biotinylated cloned DNA probe G35I. Abbreviations are the same as in Figure 1.

bands observed in RFLP patterns of strain FDB MLO corresponded to DNA fragments of the same sizes in RFLP patterns of DNA from strain G, the overall RFLP patterns differed among the three MLOs. A prominent DNA fragment of about 5,800 bp was detected in digested DNA from strain FDU using both probes G35I and G39I. No corresponding DNA fragment of this size was observed in the RFLP patterns of DNAs from strains FDB or G.

Similarity coefficients, a reflection of genetic relatedness calculated on the basis of RFLP patterns obtained with all probe/enzyme combinations, were 0.13 between strains FDU and FDB, 0.09 between strains FDU and G, and 0.38 between strains FDB and G.

DISCUSSION

In this study, pathogens associated with *flavescence dorée* and southern European grapevine yellows were compared with one another. That the pathogens had infected grapevine was indicated by their dodder transmission from grapevines to periwinkle, while source and recipient plants were protected from feeding by possible insect vectors. Evidence that the pathogens were MLOs was provided by data in the present and other work (R. E. Davis, *unpublished*) in which MLO-specific probes consisting of known MLO DNA sequences yielded positive dot hybridizations against nucleic acid extracted from infected plants including grapevine. As in previous work with other MLOs (9–11,14–17), it was possible to recognize similarities and differences among the MLO strains on the basis of results from dot hybridizations of cloned DNA probes against nucleic acid samples from infected plants. As expected, relatedness among the various MLOs was more readily revealed when hybridization reactions were carried out under conditions of moderate stringency, whereas hybridizations carried out under conditions of high stringency tended to distinguish the MLO strains from one another. Thus, although sharing of some nucleotide sequence homologies was evident, strains FDU, FDB, and G were distinguished from one another, as well as from American aster yellows MLO strain AY1, since each displayed an individually distinct pattern of dot hybridizations against the six cloned MLO DNA probes employed. For example, use of probes G20AI, pAY18, and pAY22 in high-stringency dot hybridizations was sufficient to distinguish among all four MLOs.

MLO strains FDU, FDB, and G were also distinguished from one another by RFLP analyses of chromosomal DNA. However, strains FDB and G appeared to share more similarity with one another than either did with strain FDU. Although the data indicated sharing of nucleotide sequence homologies among the three MLOs from Italy, results from the present RFLP analyses as well as data from enzymatic amplification of DNA (J. P. Prince and R. E. Davis, *unpublished*) are consistent with the concept that strains FDB and G are more closely related to one another than to strain FDU. Based on comparisons with similarity coefficients previously reported between distinct MLO strain clusters and strain "types" (subclusters) within a cluster (16,17), it appears that strains FDB and G belong to a single genomic cluster, and that strain FDU belongs to a separate cluster. This interpretation is confirmed by data from studies indicating that strains FDB and G are related to aster yellows MLO and that strain FDU is affiliated with a different MLO strain cluster (12; J. P. Prince and R. E. Davis, *unpublished*).

The name *bois noir* was proposed for a grapevine disease in which the symptoms are nearly identical to those of *flavescence dorée* (FD), but the causal agent of BN is not transmitted by the leafhopper *S. titanus*, vector of the FD pathogen in France (6,7). In northern and southern Italy, grapevine yellows diseases have been distinguished as FD disease in northern Italy and as southern European grapevine yellows disease in southern Italy (1). Biological criteria have traditionally been used to distinguish these and other MLO-associated diseases, and to identify the MLOs, because more direct methods for investigating the possible relationships among the various grapevine yellows pathogens have largely been lacking. It is interesting that the distinction in nomenclature between grapevine yellows diseases in Italy is consistent with the molecular genetic distinctions observed between MLO strains FDU and FDB in the present study. However, an overlap in the geographical ranges of these two strain types would be consistent with the occurrence in northern Italy of a MLO (strain G) related to southern European grapevine yellows MLO FDB. It thus seems possible that both *flavescence dorée* disease and southern European grapevine yellows MLO strains might be present in grapevines in northern Italy.

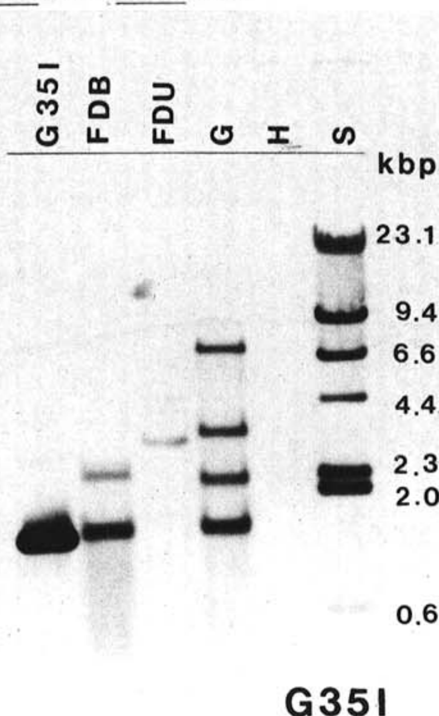

Fig. 3. RFLP analysis of chromosomal DNA of MLO strains associated with *flavescence dorée* (FDU), southern European grapevine yellows disease (FDB), or Italian periwinkle virescence (G). DNA from healthy or MLO-infected plants was doubly digested with *Eco*RI and *Hin*dIII. Hybridization was conducted at 50 C using biotinylated cloned DNA probe G35I. Lane G35I contains the probe DNA. Remaining abbreviations are the same as in Figure 1.

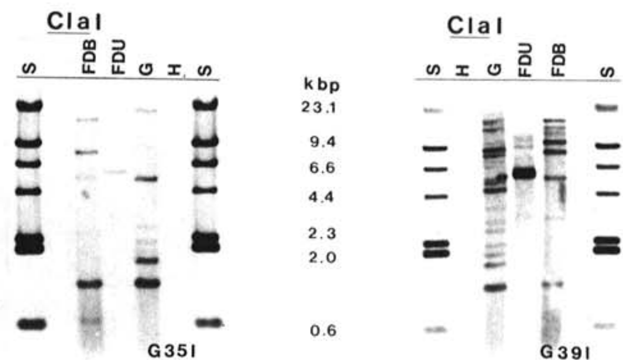

Fig. 4. RFLP analysis of chromosomal DNA of MLO strains associated with *flavescence dorée* (FDU), southern European grapevine yellows disease (FDB), or Italian periwinkle virescence (G). DNA from healthy or MLO-infected plants was digested with restriction endonuclease *Cla*I. Hybridizations were conducted at 42 C using biotinylated cloned DNA probes G35I (left) and G39I (right). Abbreviations are the same as in Figure 1.

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