# Characterization of Mycoplasmalike Organisms from *Fraxinus, Syringa,* and Associated Plants from Geographically Diverse Sites

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## ABSTRACT

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Mycoplasmalike organisms (MLOs) in six species of ash (*Fraxinus*) and lilac (*Syringa*) at 13 locations from southern Quebec and Massachusetts to Zion National Park, Utah, were detected by the DAPI (4′-6-diamidino-2-phenylindole·2HCl) fluorescence test. Relatedness of these MLOs to one another was established through dot hybridization of DNA samples from diseased plants with four ash yellows (AshY)-specific DNA probes and through immunofluorescence microscopy with an AshY-specific monoclonal antibody. In a search for possible alternative plant hosts of the AshY agent, the DAPI test was utilized to detect MLOs in 13 other species growing in the vicinity of diseased ash in central New York State and in two species in Zion National Park. These species were (asterisks indicate first record of microscopic detection of MLOs) **Apocynum cannabinum,* **Asclepias syriaca, Aster novae-angliae,* **Carya cordiformis,* **Cornus racemosa,* **Chrysopsis villosa,* **Chrysothamnus nauseosus,* **Epilobium ciliatum,* **Lotus corniculatus, Prunus virginiana, Salix* sp., **Solidago rugosa,* and **Spiraea tomentosa.* With the exception of *P. virginiana,* which contained an X-disease MLO, none of these species was found to be diseased at more than three of the 24 sites of AshY occurrence that were surveyed. Diseased phloem of 10 of these species was tested with the AshY-specific monoclonal antibody and did not react with it. A 1.2-kb fragment of DNA of the 16S ribosomal RNA gene was amplified by polymerase chain reaction from each of four MLO strains from ash and lilac, one strain each from *A. syriaca, C. racemosa, S. rugosa,* and *S. tomentosa,* and three reference strains from other sources, maintained in periwinkle (*Catharanthus roseus*). Restriction fragments obtained by digestion of the amplified products with enzymes *Alu*I, *Kpn*I, and *Mse*I were similar for the ash and lilac MLOs and differentiated them from the others tested. The MLOs detected in *A. novae-angliae, C. racemosa,* and *L. corniculatus* were related to members of the aster yellows MLO group on the basis of reaction with an aster yellows-specific monoclonal antibody. This finding for *C. racemosa* was supported by results of restriction enzyme analysis of the 16S ribosomal DNA fragment. To date, *Syringa* spp. are the only known alternative hosts of AshY MLOs.

Ash yellows (AshY) and lilac witches'-broom (LWB) (18,20, 36,37) are believed to be caused by closely related mycoplasmalike organisms (MLOs) (19). *Fraxinus americana* L. (white ash) and *F. pennsylvanica* Marsh. (green ash) affected by AshY occur widely in the eastern United States and southeastern Canada (35,50). Additional ash species with AshY and many lilac (*Syringa* spp.) cultivars with LWB have been found in arboreta (19). *F. velutina* Torr. (velvet ash) and its variety *glabra* (Arizona or Modesto ash) infected with MLOs have been found in three southwestern states (4,50). Symptoms associated with MLO infection differ among species of ash and lilac (4,10,17,38,47,48,51). White ash usually sustains progressive loss of vitality leading to dieback (37). Green ash sustains growth loss (47) that is often not accompanied by dieback. Velvet ash does not sustain significant growth loss when infected with AshY MLOs (47).

Ash- and lilac-inhabiting MLOs in New York State, Massachusetts, and Ottawa, Canada, were shown to be closely related with an AshY-specific cloned DNA probe derived from the New York MLO strain AshY1 (8,19). The relationship of MLOs in ash from central and western areas of the United States to those in the East was not known. If all MLOs inhabiting ash and lilac are closely related, they may be regarded as strains of one widely distributed pathogen. If different MLOs cause similar symptoms in ash and lilac, a new etiological concept of AshY and LWB would be needed, and epidemiological research on these diseases would have to take pathogen diversity into account.

No reports have appeared about plant hosts of AshY or LWB MLOs other than ash, lilac, and herbaceous species to which these MLOs were transmitted by dodder (19,36). If additional species are infected in nature, they could be important in the epidemiology of AshY or LWB by serving as substrates for pathogen increase. Hosts in multiple genera are involved in the epidemiology of some other diseases caused by MLOs, notably lethal yellowing of palms and aster yellows (9,16,40).

Many MLOs can be detected and genetic interrelatedness among strains determined by means of DNA-DNA hybridizations, amplifications of particular DNA sequences by polymerase chain reaction (PCR), restriction enzyme analyses of total MLO DNA or of PCR products, and immunological tests (1,25,44, and references cited therein). Groups of MLO strains have been identified on the basis of DNA homologies (1,25,26,44), and AshY MLOs have been found to comprise a genetically distinct cluster. This finding was based on studies of MLO strains maintained in periwinkle (*Catharanthus roseus* (L.) G. Don) (8,25,44) or found in

TABLE 1. Hybridization of DNA from mycoplasmalike organism (MLO)-infected *Fraxinus* and *Syringa* spp. from different locations with ash yellows (AshY)-specific DNA probes[a]

| Species | Geographic location | Signal strength[b] with DNA probe | | | |
|---|---|---|---|---|---|
| | | AA13I | AA82I | AA157I | AA176I |
| *F. americana* L. | Alto, MI | W | + | W | + |
| | Woodville, OH | W | + | W | + |
| | Ithaca, NY | + | ++ | + | ++ |
| | Tully, NY | + | ++ | + | ++ |
| | Verbank, NY | + | ND | + | ND |
| | North East, PA | − | + | W | + |
| | Gatineau Park, Quebec | ++ | +++ | + | ++ |
| *F. bungeana* A. DC. | Jamaica Plain, MA | + | ND | ++ | ND |
| *F. velutina* Torr. | Zion National Park, UT | W | + | W | W |
| *S.* × *josiflexa* Preston ex Pringle 'Royalty' | Jamaica Plain, MA | + | ND | + | ND |
| *S.* × *prestoniae* McKelv. 'Dorcas' | Jamaica Plain, MA | W | W | − | W |

[a] Data were extracted from three experiments. In each experiment, DNA samples from perwinkle plants singly infected with MLO strains AshY1 and a *Prunus*-X strain were included as positive and negative diseased standards, respectively, to verify specificity of the AA probes. A complete set of samples was also incubated with the broadly hybridizing MLO probe pBB115 to establish whether any MLO DNA could be detected. DNA from healthy *F. americana* and *S. vulgaris*, utilized as controls, did not hybridize with pBB115 or AA probes.
[b] − = No signal; W = weak positive; + to +++ = positive; and ND = no data.

other plants or insects (44). LWB MLOs were found to be closely related to AshY MLOs on the basis of dodder and graft transmissibility to and from ash; similarity of symptoms induced by AshY and LWB MLOs in ash, lilac, and periwinkle; and DNA homology (19).

The research described here expands the known geographic and botanical scope of interrelatedness of AshY and LWB MLOs, as determined through DNA-DNA hybridizations with four AshY-specific probes (8), immunofluorescence tests with a monoclonal antibody raised against MLO strain AshY2 (15), and restriction fragment length polymorphism (RFLP) analyses of PCR-amplified fragments of the 16S ribosomal RNA genes of these MLOs (31). In addition, we report the discovery of several MLO strains in plants other than ash or lilac on sites of AshY occurrence. Our objectives were to determine the relatedness of MLOs in several ash and lilac species at diverse geographic locations and to determine whether AshY MLOs commonly occur in plants of other species growing in the vicinity of MLO-infected ash. Abstracts describing parts of the work have appeared (14,15,48).

## MATERIALS AND METHODS

The general scheme of this work was to collect specimens from symptomatic and healthy plants, to test for MLO infection with the DAPI (4′,6-diamidino-2-phenylindole·2HCl) fluorescence procedure (45,49), and then to utilize one or more of the following methods to confirm the presence of MLOs and attempt their identification: 1) extract DNA from diseased and healthy plants and perform dot hybridizations with cloned AshY-specific and nonspecific MLO-detecting DNA probes; 2) extract DNA as above, amplify fragments of MLO DNA by PCR, and perform RFLP analyses of the products; and 3) perform immunofluorescence microscopy procedures with two monoclonal antibodies, one specific for AshY MLOs and the other for certain aster yellows MLOs.

**Sources and initial detection of MLOs.** Ash and lilac specimens were collected at 13 locations from Massachusetts and southern Quebec to Utah (Tables 1 and 2). Small roots or blocks of conductive phloem (innermost bark) of large roots were preserved in 2.5% aqueous glutaraldehyde for the DAPI fluorescence test (49). If MLOs were detected with DAPI, fine roots or conductive phloem of large roots were sampled for DNA extraction and immunofluorescence studies. Prior work had indicated that MLOs could be detected by DNA hybridization or immunofluorescence only if they could be detected with DAPI (46; H. M. Griffiths and W. A. Sinclair, *unpublished*).

A search was made for plants other than ash with symptoms that might be caused by MLOs at 24 sites of AshY occurrence in central New York State and Zion National Park, Utah. The sites varied in area from approximately 0.1 to 2 ha. Symptoms included stunted shoots or leaves, suppressed flowering, chlorosis, rosettes, witches'-brooms, and abnormally abundant basal sprouts. Symptomatic and apparently healthy plants of each affected species were sampled from the same sites (small plants collected whole), and specimens were preserved for DAPI tests.

TABLE 2. Reaction of DAPI[a] and a monoclonal antibody raised against ash yellows mycoplasmalike organism (MLO) strain AshY2 with phloem of periwinkle containing known strains of MLOs and with phloem of *Fraxinus* and *Syringa*

| Plant species | MLO strain or locality of specimen collection[b] | DAPI reaction[c] | Immunofluorescence reaction[c] |
|---|---|---|---|
| *Catharanthus roseus* (L.) G. Don | Ash yellows, AshY1 and AshY2 | + | + |
| | Lilac witches'-broom, LWB1 and LWB2 | + | + |
| | Aster yellows, AY1 and NJAY | + | − |
| | Tomato bigbud, BB | + | − |
| | Clover phyllody, CPh | ND | − |
| | Peach X, CX | + | − |
| | Elm yellows, EY1 and EY3 | + | − |
| | Grapevine yellows | ND | − |
| | Sweet potato witches'-broom | ND | − |
| | Beet leafhopper-transmitted virescence agent, VR | + | − |
| *F. americana* L. | Ann Arbor, MI (2) | + | + |
| | Woodville, OH (2) | + | + |
| | Geneva, OH (2) | + | + |
| | Ithaca, NY (2) | + | + |
| | Verbank, NY (1) | + | + |
| | North East, PA (2) | + | + |
| *F. pennsylvanica* Marsh. | Ann Arbor, MI (1) | + | + |
| | Minneapolis-St. Paul, MN (4) | + | +[d] |
| | Ithaca, NY (2) | + | + |
| | Union Springs, NY (2) | + | + |
| *F. velutina* Torr. | Zion National Park, UT (5) | + | +[e] |
| *S.* × *prestoniae* McKelv. | Ithaca, NY (1) | + | + |

[a] 4′-6-Diamidino-2-phenylindole·2 HCl.
[b] Numbers of samples examined are in parentheses. Healthy specimens of perwinkle, all ash species, and common lilac were examined and did not react with the antibody.
[c] − = No reaction; + = positive; and ND = no data.
[d] Two of four samples positive.
[e] Two of five samples positive.

Plants or samples in which MLOs were detected with DAPI were retained for DNA extraction and immunofluorescence tests.

MLO-infected plants from the New York sites were transplanted into pots and, after a quarantine period, were maintained in a screened greenhouse where insects were controlled by periodic fumigation. For MLO-infected plants too large to move (e.g., *Salix* sp.), diseased shoots were propagated by cuttings or grafted to healthy plants of the same species. MLO transmission by dodder (*Cuscuta subinclusa* Dur. & Hilg.) from collected plants into seed-propagated periwinkle was attempted as described previously (3). Transmissions were verified by means of the DAPI test.

Reference strains of MLOs (strain designations in parentheses) in periwinkle plants had the following origins: ash yellows MLOs (AshY1 and AshY2) from white ash growing at Ithaca and Enfield, New York, respectively; a Maryland strain of aster yellows MLO (AY1) from periwinkle; a New Jersey strain of aster yellows MLO (NJAY) from lettuce; beet leafhopper-transmitted virescence agent (VR) from periwinkle in California by G. N. Oldfield; tomato bigbud (BB) MLO from Arkansas by J. L. Dale; clover phyllody (CPh) MLO and a strain of *Prunus*-X MLO (CX) from Ontario by L. N. Chiykowski; two strains of elm yellows MLOs (EY1 and EY3) from Ithaca, New York; lilac witches'-broom MLOs from *Syringa* × *prestoniae* McKelv. 'Hiawatha' growing at Ithaca, New York (LWB1), and from *S.* × *josiflexa* Preston 'Royalty' growing at Ottawa, Ontario (LWB2).

**Dot hybridizations.** For samples of ash, lilac, and periwinkle containing AshY or LWB MLOs (Table 1), DNA was extracted from 0.3-g portions of MLO-infected or healthy tissues as described previously (7,24), applied to nylon (Nylon-1, GIBCO BRL, Grand Island, NY) or nitrocellulose (Schleicher and Schuell, Keene, NH) membranes, and baked 2 h at 80 C. The amount of DNA on each spot was not standardized. DNA probes, prepared as described previously (8,27), were labeled by nick translation of cloned recombinant plasmids with biotin-7-dATP according to instructions of the manufacturer (GIBCO BRL). Samples were hybridized by the methods of Lee and Davis (24) at 42 C with probe pBB115, which hybridizes with DNA of many MLOs (27); and at 52 C with AshY-specific probes AA13I, AA82I, AA157I, and AA176I (8). The AshY probes had been selected for their ability to hybridize, under the stated conditions, with DNA of AshY MLOs but not with DNA of mollicutes associated with 11 other plant diseases (8). Hybridization was detected with the NBT/BCIP colorimetric method in the BluGENE detection system (GIBCO BRL). DNA samples from appropriate healthy plants and from periwinkles singly infected with AshY and CX MLOs were included in each experiment to verify specificity of the AA probes.

For studies of MLOs transmitted to periwinkle from wild plants other than ash or lilac, total DNA was extracted by the method of Lee et al (28) from healthy periwinkles and from those containing reference strains or MLOs from wild plants. Concentration of total DNA in each sample was estimated by comparative agarose gel electrophoresis (42) with appropriate standards. Membranes for dot hybridizations were prepared with 1.0–1.5 g of total DNA per sample. Probes (100–150 ng of DNA) were labeled with $^{32}$P-dCTP by using random oligoprimers (Amersham Corp., Arlington Heights, IL). Membranes were prehybridized for at least 4 h in the presence of 50% formamide at 42 C (23). Labeled probe was added, and hybridization was performed for at least 16 h at 42 C. Posthybridization wash conditions were 2× SSC (1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate), 0.1% SDS (sodium dodecyl sulfate) at 37 C for 10 min and again at 37 C for 30 min; and then 0.1× SSC, 0.1% SDS at 65 C for 30 min (23). Membranes were first hybridized with probe pCP67, which hybridizes with DNA of various MLOs (28), stripped as described by Sambrook et al (42), hybridized with AshY-specific probe AA13I or AA176I, stripped again, and hybridized with aster yellows-specific probes pAY24 and pBB24 (24,27). Hybridization signals were detected by exposing Kodak X-OMAT AR X-ray film with two intensifying screens at −80 C for 2–5 days. Films were developed with an X-OMAT automatic developer (Kodak, Rochester, NY). MLO strain HD1 from hemp dogbane was omitted from these and the PCR-RFLP experiments described below, because it was obtained after the work with other MLO strains in periwinkle was complete.

**PCR-RFLP analyses.** The method of Lee et al (28) was used to extract DNA from periwinkle plants. DNA concentration was estimated by comparative agarose gel electrophoresis (42) and adjusted to 20 ng/μl. PCR analyses were performed with all samples with primer set R16F2R2 (31) on the basis of a portion of an MLO 16S ribosomal gene sequence (32). This primer pair amplifies a 1.2-kb rDNA fragment from all mollicutes tested but does not amplify DNA from healthy plants (31). Two additional sets of PCR primers, AY18p-AY18m and AY19p-AY19m, which specifically prime the amplifications of genomic DNA sequences from certain aster yellows MLOs (26,43), were utilized in an attempt to identify an MLO strain detected in gray dogwood.

PCR mixtures were prepared as described previously (30,31,43); 50-μl final volumes were used in a Thermal Cycler 9600 (Perkin-Elmer Cetus, Norwalk, CT). The mixtures were subjected to initial denaturation at 94 C for 75 s and then to 35 cycles as follows: 10 s at 94 C for denaturation, 58 s at 50 C for annealing, and 128 s at 72 C for polymer extension. During the final cycle, 6 min 5 s were allowed for extension. Negative control tubes for each reaction included DNA from healthy plants of each species and a tube with sterile distilled water in place of template DNA. A positive control tube contained amplifiable MLO DNA from periwinkle infected with MLO strain AY1.

PCR products were detected and their sizes estimated by electrophoresis of 5 μl of the reaction mixture with appropriate molecular weight standards in 0.7% agarose gel in TBE buffer (45 mM Tris-borate, 1 mM EDTA, pH 8.0) at 5 V/cm. Samples of the product (50 ng of DNA per microliter) were then digested at 37 C for at least 2 h with restriction enzymes *Alu*I, *Kpn*I (Boehringer Mannheim, Indianapolis, IN), or *Mse*I (New England Biolabs, Beverly, MA). Each digest contained 5 units of enzyme per microgram of DNA in the presence of spermidine (40 mM) in a 20-μl reaction volume. The PCR product amplified from MLO strain GD1 was also digested with *Hha*I (New England Biolabs) using similar procedures. Digests were analyzed on 4% agarose gels (3:1, wide range-standard agarose, Sigma Chemical Co., St. Louis, MO) in TAE buffer (40 mM Tris-acetate, 1 mM EDTA) at 3 V/cm. An *Hae*III digest of pBR322 (Sigma) and the 1 Kb DNA ladder of GIBCO BRL provided molecular weight standards. After electrophoresis, gels were stained with ethidium bromide (2 μg/ml), destained in distilled water for 30 min, and photographed.

**Production of AshY-specific monoclonal antibodies.** For antigen preparation, 50 g of small leaves or young stems of periwinkles infected with MLO strain AshY2 were triturated in an Omni-Mixer (Sorvall, Norwalk, CT) at low speed for 2–3 min. The mixture was washed with distilled water and squeezed through two layers of cheesecloth, which removed much of the parenchyma. The remaining tissues were placed in 200 ml of enzyme solution containing 0.6 M mannitol, 30 mM 3-(4-morpholino)-propanesulfonic acid (MOPS), 1 mM calcium chloride, and 15 units of pectinase (per milliliter), pH 5.4, and incubated at 4 C overnight. The tissues were then transferred to 300 ml of cold isolation medium, which was the same as the enzyme solution except without pectinase, and the pH was adjusted to 7.2. The suspension was stirred at 4 C until vascular elements were completely separated from the surrounding tissues; it was then clarified by centrifugation (8,000 *g*, 10 min). The supernatant was subjected to additional centrifugation (16,000 *g*, 30 min) to precipitate the MLOs. The pellet was suspended in isolation medium and pelleted again at 16,000 *g*. This pellet, containing partially purified AshY MLOs, was suspended in 0.5 ml of phosphate-buffered saline (PBS) (pH 7.0), homogenized by ultrasonication, and used as antigen for immunizing mice.

Six-week-old BALB/c mice were each immunized by two intraperitoneal injections, 7 days apart, with 100 μl of antigen preparation mixed with an equal volume of Freund's complete adjuvant. Thirty days after the second injection, the mice were given booster injections on two consecutive days with the same

volume of antigen preparation, except without adjuvant. The mice were sacrificed 3 days later, and spleen cells were collected for cell fusion.

The procedure for fusion with mouse myeloma cells (P3/NSI/1-Ag4-1) was that of Lin and Chen (33,34). Hybridomas were selected with HAT medium (hypoxanthine, aminopterin, and thymidine) (33). Indirect enzyme-linked immunosorbent assay (ELISA) was employed to select specific antibody-producing hybridomas by screening the supernatant of hybridomas against both diseased and healthy periwinkle plant preparations. Periwinkle materials for coating ELISA plates were prepared as follows: small leaves or leaf veins (10 g) were ground in isolation medium; after low-speed (8,000 *g*) and high-speed (16,000 *g*) centrifugation, the pellet was suspended in 100 ml of coating buffer (0.05 M carbonate-bicarbonate buffer, pH 9.6) and treated by ultrasonication for 30 s. Hybridomas producing specific antibodies to diseased periwinkle preparation were selected and subcultured. Monoclonal cell lines were obtained by limited dilution (33).

**Isotype and specificity of monoclonal antibodies.** The immunoglobulin isotype of each monoclonal antibody was determined with the ISOTYPE Ab-STAT-I&II Kit (SangStat Medical Corporation, Menlo Park, CA). Specificity of monoclonal antibodies was tested by means of indirect ELISA, performed as described previously (33,34), and by immunofluorescence microscopy. For ELISA, antigen preparations were obtained from periwinkles singly infected with two strains of AshY MLOs; from two strains of aster yellows MLOs (Maryland and New Jersey origins); from MLOs associated with clover phyllody, elm yellows, grapevine yellows, and sweet potato witches'-broom; from *Spiroplasma citri;* and from healthy and MLO-infected white ash.

**Immunofluorescence microscopy.** Monoclonal antibodies produced against MLO strain AshY2, as described above, and a New Jersey strain of aster yellows MLO (NJAY) (33,34) were used as described by Jiang et al (22) to detect MLOs in plant materials and to verify specificity of the AshY antibody. The NJAY antibody is known to react with certain strains of aster yellows MLOs but not with strain AY1 or with any of six other MLO strains representing different diseases. An undiluted supernatant containing AshY MLO antibody from hybridoma culture was used. Purified NJAY antibody, adjusted to 200 μg of protein per milliliter in PBS, pH 7.4 (34), was diluted (1:5) in PBS. To ascertain the specificity of the AshY MLO antibody, transverse or longitudinal sections were cut by hand or with a freezing microtome from stems of periwinkle singly infected with *S. citri* or with 14 strains of MLOs representing diverse plant and geographic sources. Healthy and diseased ash and lilac samples treated with AshY antibody were tested similarly. Plants other than ash and lilac were tested with both AshY and NJAY antibodies. In each test, the secondary antibody was fluorescein isothiocyanate-conjugated goat anti-mouse (IgG + IgM), $F(ab')_2$ fragment (Boehringer Mannheim). Preparations were viewed with microscopes equipped for fluorescence with 460–485-nm excitation filters, 505-nm dichroic mirrors, and 515-nm barrier filters.

## RESULTS

**Dot hybridizations with DNA from ash and lilac.** All four AshY-specific probes hybridized with each of the two AshY and the two LWB MLO strains in periwinkle (data not shown). Table 1 shows the results obtained from hybridization of DNA from MLO-infected *Fraxinus* and *Syringa* spp. from different locations with AshY-specific DNA probes. Probe AA13I hybridized with eight of the nine ash samples and with both lilac samples. Probes AA82I and AA176I hybridized with all ash samples tested and with one lilac sample. All ash samples and one lilac sample hybridized with pAA157I. In each experiment, DNA samples from periwinkle plants singly infected with MLO strains AshY1 and CX were included as positive and negative diseased standards, respectively, to verify specificity of the AA probes. Hybridization of the broadly hybridizing probe pBB115 with DNA from all sources mentioned above confirmed that experimental conditions were adequate for hybridization. These results indicate that the MLOs detected in ash and lilac are closely related to one another and to the strains of AshY and LWB MLOs maintained in periwinkle.

**Monoclonal antibody isotype and specificity.** Three stable hybridomas secreting antibodies specific to AshY MLO-infected periwinkles but not to healthy periwinkles were obtained from a single fusion. Three monoclonal cell lines were selected from these original clones. When isotyped, all three monoclonal antibodies were determined to belong to the IgM class. One antibody was selected for further work. In indirect ELISA with this antibody, preparations from periwinkle containing MLO strains AshY1 and AshY2 gave optical density values (at 490 nm) of 1.301 and 0.819, respectively. A preparation from AshY-affected white ash gave a value of 0.411. Healthy ash and periwinkle and periwinkles infected with *S. citri* or with any of the six other

TABLE 3. Mycoplasmalike organism (MLO) detection by DAPI[a] tests or immunofluorescence tests with monoclonal antibodies in wild plants other than ash or lilac growing on sites of ash yellows occurrence in New York State and Utah

| Location | Plant[b] | Method of MLO detection[c] | | |
|---|---|---|---|---|
| | | | Monoclonal antibody against MLO strain | |
| | | DAPI | AshY2 | NJAY |
| Central New York | **Apocynum cannabinum* L. (hemp dogbane) | + | ND[d] | ND[d] |
| | **Asclepias syriaca* L. (common milkweed) | + | − | − |
| | *Aster novae-angliae* L. (New England aster) | + | − | + |
| | **Carya cordiformis* (Wangenh.) K. Koch (bitternut hickory) | + | ND | ND |
| | **Cornus racemosa* Lam. (gray dogwood) | + | − | + |
| | **Epilobium ciliatum* Raf. (northern willowherb) | + | ND | ND |
| | **Lotus corniculatus* L. (bird's-foot trefoil) | + | − | + |
| | *Prunus virginiana* L. (chokecherry) | + | − | ND |
| | *Salix* sp. (unidentified willow) | + | − | ND |
| | **Solidago rugosa* Mill. (goldenrod) | + | − | − |
| | **Spiraea tomentosa* L. (spirea, hardhack, steeplebush) | + | − | − |
| Zion National Park, UT | **Chrysopsis villosa* (Pursch) Nutt. (hairy golden aster) | + | − | − |
| | **Chrysothamnus nauseosus* (Pall. ex Pursh) Britton (rabbitbrush) | + | − | − |

[a] 4′-6-Diamidino-2-phenylindole·2 HCl.

[b] * = Species for which no previous published record of microscopic detection of MLOs was found. For controls, healthy plants of each species, collected on the same sites, were tested with DAPI and AshY or NJAY monoclonal antibody; all gave negative reactions except *A. cannabinum*, in which both healthy (DAPI-negative) and diseased phloem reacted with goat anti-mouse fluorescein isothiocyanate conjugate.

[c] − = No reaction; + = positive; and ND = no data.

[d] MLOs from *A. cannabinum*, transmitted to periwinkle after the completion of other work described here, did not react with AshY or NJAY antibodies.

MLO strains tested all gave values of <0.1.

**MLO detection in ash and lilac by immunofluorescence.** When tested against 14 MLO strains and *S. citri* in periwinkle, the selected monoclonal antibody reacted with the four MLO strains that originated in ash or lilac but not with any of 10 other strains of MLOs representing eight diseases (Table 2). MLO-infected (DAPI-positive) ash and lilac specimens representing four species and nine locations across the range of AshY were then tested. Positive reactions were obtained with samples from every species and location (Table 2). Phloem of periwinkle plants infected with MLOs other than those of AshY or LWB and control specimens from healthy (DAPI-negative) plants of periwinkle, each ash species, and common lilac (*S. vulgaris* L.) did not react with the antibody.

**MLOs in wild plants other than ash and lilac.** Plants, other than ash, most consistently found infected by MLOs in central New York were *Prunus virginiana* L. and *Ulmus americana* L., which displayed characteristic symptoms of eastern X-disease and elm yellows, respectively. The elm disease was not studied further, because MLOs associated with elm yellows were known to differ from those of AshY (7,8,29). MLOs were detected by means of the DAPI test in symptomatic plants of 10 additional species, each found on one or more sites of AshY occurrence in central New York (Table 3). None of these species was found diseased at more than three locations, and only symptomatic goldenrod and milkweed were abundant at any site. MLOs were also detected by means of DAPI tests in one plant of each of two species other than ash in Zion Canyon, Zion National Park (Table 3). No prior published records of yellows-type diseases were located for six of the species found diseased in New York State or either of the two species found in Utah. Our microscopic detection of MLOs is the first on record for 10 species.

Symptoms associated with MLO infection of the herbaceous species were variable and included stunting of shoots and leaves; chlorosis, more severe between veins and toward leaf margins than along the veins; and floral suppression or sterility. Entire plants were affected. Bird's-foot trefoil, a legume, was exceptional in remaining green but stunted. Among the woody species, gray dogwood and spirea displayed stunted leaves; abnormally short, upright spindly shoots; and precocious opening of axillary buds, all of which contributed to a dwarfed, bushy form of entire plants. Diseased willow had numerous small witches'-brooms on otherwise normal-appearing branches. These brooms died during dormant periods. MLOs could be detected with DAPI only within the brooms. Rooted cuttings from healthy-appearing branches on diseased willows grew normally and tested negative with DAPI. A single bitternut hickory was found with a witches'-broom approximately 2 m tall on the trunk 8 m above ground level. MLOs were detected with DAPI in phloem from a buttress root of this tree.

Five MLO strains were transmitted by dodder from wild plants to periwinkle. The wild plants and the designations given to these MLO strains in periwinkle were goldenrod, GR1; milkweed, MW1; gray dogwood, GD1; hemp dogbane, HD1; and spirea, SP1. Attempts to transmit MLOs from chokecherry, New England aster, northern willowherb, and willow to periwinkle were unsuccessful. MLO-infected bird's-foot trefoil died soon after transplanting, thus precluding transmission.

Dot hybridizations with $^{32}$P-labeled DNA probes were performed with DNA extracted from DAPI-positive periwinkle plants containing MLO strains AshY1, AshY2, AY1, CX, GD1, GR1, MW1, and SP1. All of these samples hybridized with the broad-spectrum probe pCP67. AshY-specific probes AA13I and AA176I hybridized only with DNA from plants containing AshY1 or AshY2, while probes pAY24 and pBB24, which detect aster yellows MLOs, hybridized only with DNA of strain AY1 (data not shown). Dot hybridizations were also attempted with DNA extracted from DAPI-positive bitternut hickory, chokecherry, and hairy golden aster but were unsuccessful with all probes tested.

Ten species of wild plants that gave positive reactions with DAPI gave negative reactions when tested by immunofluorescence microscopy with the monoclonal antibody raised against MLO strain AshY2 (Table 3). Bird's-foot trefoil, gray dogwood, and New England aster gave positive reactions with the aster-yellows antibody. Hemp dogbane phloem from both healthy and MLO-infected plants reacted with fluorescein isothiocyanate-labeled goat anti-mouse conjugate in the absence of MLO antibody. Periwinkle phloem infected with MLO strain HD1 from hemp dogbane did not react with AshY2 or NJAY monoclonal antibody. The single specimens of diseased bitternut hickory and northern willowherb died or were used for DNA extractions before the serological tests began.

**MLO detection and differentiation by PCR and RFLP analyses.** A 1.2-kb rDNA fragment was amplified from every MLO-infected periwinkle sample that was tested but not from healthy controls or sterilized water. RFLP profiles obtained from *Alu*I and *Mse*I

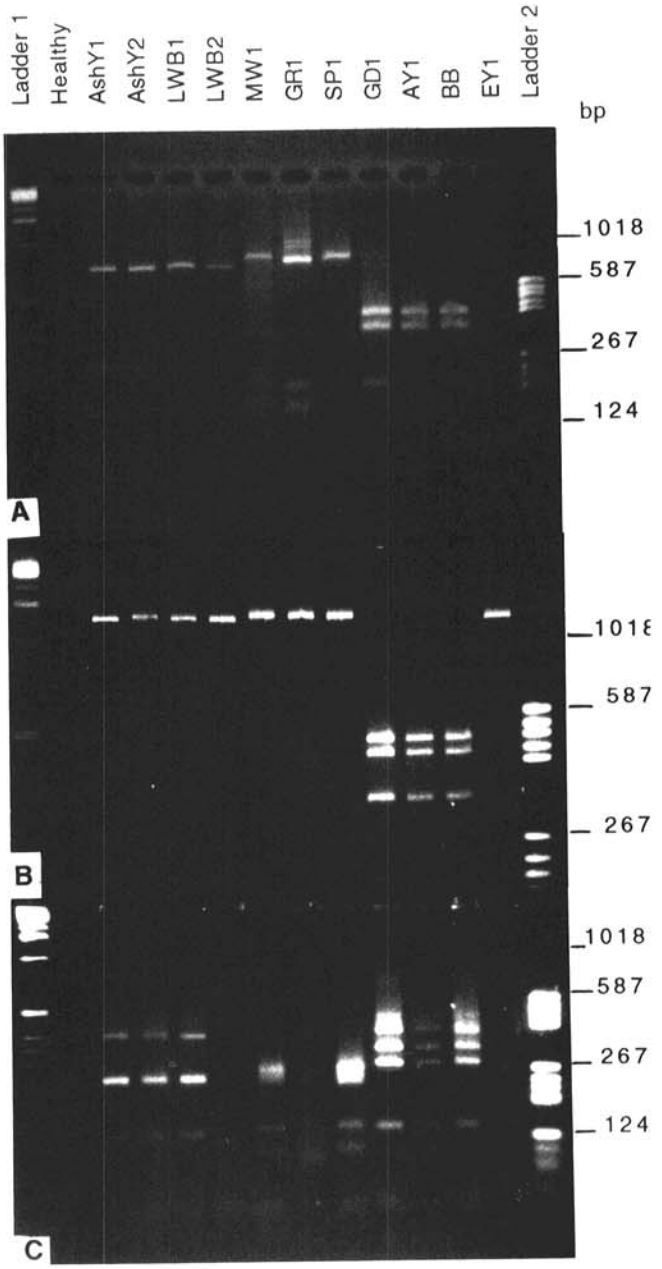


**Fig. 1.** Restriction fragment profiles obtained by electrophoresis of 16s rDNA fragments amplified by polymerase chain reactions from various mycoplasmalike organism strains in periwinkle after digestion with restriction endonuclease *Alu*I, *Kpn*I, or *Mse*I. Size standards (outside lanes) were ladder 1, the 1 Kb DNA ladder of GIBCO BRL, and ladder 2, an *Hae*III digest of pBR322. Samples, left to right, were healthy periwinkle, MLO strains AshY1, AshY2, LWB1, LWB2, MW1, GR1, SP1, GD1, AY1 (aster yellows standard), BB (tomato bigbud standard), and EY1.

digests of rDNA from all MLO strains from ash (AshY1, AshY2) and lilac (LWB1, LWB2) were identical (Fig. 1A and C). The RFLP profiles of MLO strains MW1, GD1, GR1, and SP1 after digestion with *Alu*I or *Mse*I differed from those of the ash and lilac MLOs and elm yellows MLO strain EY1. Restriction sites for *Kpn*I were not present in the 1.2-kb fragment from AshY or LWB MLOs or GR1, MW1, or SP1 (Fig 1B). The 1.2-kb product from strain GD1 from gray dogwood was cut by all three enzymes, and the restriction fragments matched those from strains AY1 and BB of the aster yellows genomic cluster. The restriction pattern obtained with *Hha*I matched that of MLO strain BB, a member of AY MLO strain type I (26), and differed from that of AY1, which is a type II strain (data not shown). No product was obtained from strain GD1 by PCR with primer set AY18 or primer set AY19.

We failed to amplify MLO DNA from bird's-foot trefoil, New England aster, or willow. DNA preparations from these plants may have contained substances that inhibited the PCR. MLO-infected and healthy chokecherry samples were processed for DNA extraction and PCR in the laboratory of the fourth author. A 1.2-kb rDNA fragment was amplified from MLO-infected plants but not from healthy plants. RFLPs obtained with *Alu*I, *Kpn*I, or *Mse*I indicated the chokecherry MLO to be similar to strain CX (data not shown).

## DISCUSSION

MLOs detected in four species of ash and two of lilac growing at 13 locations from Massachusetts and southern Quebec to Utah were found to be closely related to the New York strains AshY1 and AshY2, from which the AA probes and the AshY monoclonal antibody, respectively, were derived. RFLP analyses of the 1.2-kb 16S rDNA gene fragments from MLO strains AshY1, AshY2, LWB1, and LWB2 revealed identical patterns. These results augment the previous finding (19) of closely related MLOs associated with AshY and LWB in the northeastern United States and eastern Ontario, Canada. We propose that the MLOs in both ash and lilac be called AshY MLOs but that separate names of the diseases for the ash and lilac be maintained. Thus, both AshY and LWB are caused by MLOs in the AshY genomic cluster.

The interrelatedness of all AshY and LWB MLOs and the lack of reaction of AshY probes or the monoclonal antibody raised against AshY2 with any of 19 other MLO strains investigated or used as standards in this work support the concept that AshY MLOs comprise a genetically distinct cluster (8,25,44). These data also support the interpretation of AshY as a disease caused by one pathogen, i.e., a distinct set of closely related MLO strains. Genetic variability among AshY MLOs does occur, however, because RFLPs in genomic DNA of MLO strains infecting white ash trees in the eastern United States have been detected (39).

It is possible that AshY MLOs are transmitted only within and between ash and lilac species under natural conditions, because these MLOs were identified only in ash and lilac. None of the other diseases studied, other than elm yellows and X-disease of chokecherry, was found on more than three sites of AshY occurrence. Host limitation of AshY MLOs in nature may be associated with insect vector feeding preference rather than with resistance of a particular plant, since these MLOs have been experimentally transmitted by dodder into carrot (*Daucus carota* L.) (19) and red clover (*Trifolium pratense* L.) (W. A. Sinclair, *unpublished*). It is also possible that AshY MLOs occur in symptomless hosts, but this was not studied because practical methods for assaying many plants of numerous species were not available.

Intensity of hybridization signal with biotinylated DNA probes and intensity of fluorescence in tests with AshY antibody were both related to titer of MLO populations in ash and lilac specimens as observed during DAPI tests. Thus, the absence of hybridization signal with two samples (Table 1) was probably due to low MLO titer.

Thirteen diverse plant species, including 10 in which MLOs had not previously been detected microscopically, were found infected with MLOs on sites where AshY also occurred. Included among these were the MLOs of elm yellows and X-disease of *Prunus,* which were known to be different from AshY MLOs (8,25,30). Four previously unknown MLO strains transmitted from wild plants into periwinkle were characterized sufficiently to establish that they are not AshY or elm yellows MLOs. We have given these four new MLO strains acronyms allied with the common English names of the hosts: GR1 from goldenrod, GD1 from gray dogwood, MW1 from milkweed, and SP1 from spirea. Serial numbers are needed if two or more MLO strains associated with the same disease are studied, for example, AshY1 and AshY2.

MLO-infected milkweed and goldenrod plants both displayed dwarfing, chlorosis, and abnormal development of upright axillary shoots. Therefore, we suggest the names milkweed yellows and goldenrod yellows for these diseases. Thornberry (52) listed "Asclepias yellows" of *A. syriaca* among viral diseases, but we could not find a description associated with this name. We found milkweed yellows at three locations near Ithaca, New York, and also in Canada at Tichbourne, Ontario. A similar milkweed disease, observed in eastern Ontario as early as 1930, was described by Savile (5) and later by Gilmer (13). Gilmer presented transmission and symptomological evidence that the causal agent, then thought to be a virus, was the same as that associated with X-disease in chokecherry. We have obtained molecular data, to be presented elsewhere, that the milkweed MLO is related to members of the X-disease group.

We found goldenrod yellows at two sites in central New York. Goldenrods infected with MLOs had previously been observed in Connecticut and Maryland (R. E. Davis, *unpublished*) and Vermont (W. A. Sinclair, *unpublished*). Craft (6) found *Solidago canadensis* L. with yellows-type symptoms in central New York State and obtained immunological evidence of MLO infection. Because goldenrods are in the Asteraceae, we expected that MLO strain GR1 would be similar to aster yellows MLOs. No such relationship was indicated, however, by RFLP analysis of MLO rDNA or by reaction of GR1 with the NJAY antibody.

Since gray dogwood and spirea plants infected by MLOs were stunted compared with healthy plants, the names dogwood stunt and spirea stunt are proposed. Each disease was found at only one site. MLO infection of gray dogwood was detected previously in New York State (38) by means of Dienes' stain (J. Matteoni, *personal communication*). The MLO strain obtained from this species (strain GD1) is evidently a member of the aster yellows group on the basis of the reaction with the aster yellows antibody and rDNA RFLP analysis. The restriction pattern obtained for GD1 with *Hha*I was identical to that of type I strains in the aster yellows group (26). The lack of amplification of a PCR product from GD1 with primer sets AY18 and AY19 may reflect the fact that AY18 is fairly specific for type II aster yellows MLOs, while AY19 has primed the amplification of DNA from only two MLO strains, both of aster yellows type I. MLOs observed by Raju et al (41) in witches'-brooms of *Cornus amomum* Mill. have not been characterized.

The MLOs detected in New England aster and in bird's-foot trefoil are apparently in the aster yellows group, because they reacted with the NJAY monoclonal antibody. Freitag (12) reported leafhopper transmission of a western aster yellows agent into bird's-foot trefoil.

The disease of willow, found at two sites, fits Holmes' description of Salix yellows (21). We were able to root cuttings from brooms but were unable to obtain MLO transmission by dodder from rooted cuttings to periwinkle or to obtain a PCR product from DNA extracted from the brooms.

The newly discovered diseases in bitternut hickory and northern willowherb were not studied sufficiently to confirm that the pathogens are not AshY MLOs. The disease of northern willowherb should be studied in relation to reports of MLOs in other *Epilobium* species (2,11).

The findings conveyed here support the concept of AshY MLOs as a discrete cluster (8,44) and indicate that they may be found only in the members of the Oleaceae in nature. To date, the

known host range, apart from inoculated laboratory plants, is confined to species of *Fraxinus* and *Syringa*. The MLOs detected in plants other than ash and lilac during this research merit further study to determine additional similarities and differences with respect to well-characterized MLOs.

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