Genetic Relatedness of Mycoplasmalike Organisms Affecting Elm, Alder, and Ash in Europe and North America

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ABSTRACT

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DNA from strain ULW of the European elm witches'-broom mycoplasmalike organism (MLO) maintained in periwinkle was enriched by repeated bisbenzimide-CsCl density gradient centrifugation and cloned in *Escherichia coli.* Three cloned DNA fragments of the ULW MLO were used as probes to hybridize Southern blots of *Hin*dIII-digested DNA prepared from MLO-infected elm, alder, and ash trees and from periwinkle plants infected by various MLOs. Hybridization profiles similar to that of the ULW MLO were obtained with DNA from elm witches'-broom-affected *Ulmus minor* (*U. carpinifolia*) and *U. glabra* collected at various locations in Europe, from American strain EY1 of the elm yellows MLO, from elm yellows–affected *U. americana* and *U. parvifolia* collected in New York State and Michigan, and from alder yellows–affected *Alnus glutinosa* and *A. incana* collected in various countries of Europe. Thus, European elm witches'-broom and North American elm yellows, as well as European alder yellows, may be caused by closely related MLOs. The MLOs affecting *A. rubra* (*A. oregona*) in Washington State differ from the ULW MLO, because only one of the three ULW probes hybridized with all DNA samples from this species. Two of the probes hybridized to DNA from field-collected samples of ash yellows–diseased *Fraxinus americana* and from a periwinkle-maintained strain of the ash yellows MLO. However, the sizes of the ash yellows MLO fragments hybridizing to the probes differed from those of the probes. Restriction fragment length polymorphisms were observed among the ash yellows samples. More distantly related to the MLOs occurring in elm and in alder in Europe than the ash yellows MLOs were eight periwinkle-maintained MLO strains from various woody and herbaceous hosts.

Mycoplasmalike organisms (MLOs) induce diseases in many forest and landscape tree species (24). In eastern and central North America, five native elm species (*Ulmus americana* L., *U. rubra* Mühl., *U. alata* Michx., *U. serotina* Sarg., and *U. crassifolia* Nutt.) are affected by elm yellows (EY). This disease, formerly called elm phloem necrosis, is lethal to the five species affected and is particularly important in *U. americana,* the white or American elm (5). Until the 1980s, EY was considered to be restricted to North America (25,32). In Europe, the MLO-associated elm witches'-broom disease of *U. minor* Mill. (*U. carpinifolia* Gled., European field elm) was observed in former Czechoslovakia, Italy, and France (4,27; G. Morvan, *personal communication*). Conti et al (6) described symptoms from Italy similar to those reported for graft-inoculated European elms in the United States (5). Although molecular evidence of relatedness of Italian and North American elm-infecting MLOs was not presented, the disease occurring in Italy was referred to as elm yellows (6). Recent nucleic acid hybridization analyses revealed that strain EY1 (previously designated as EY) of the EY pathogen in periwinkle (*Catharanthus roseus* (L.) G. Don) is only distantly related to the MLOs associated with diseases of herbaceous hosts such as aster yellows, big bud of solanaceous plants, clover phyllody, clover proliferation, maize bushy stunt, and others (2,8,17,19,21,22). However, the EY1 MLO seems to be related to the MLO causing flavescence dorée of grapevine in France (7).

Another mycoplasmal disease of trees, which has only recently been discovered, is alder yellows (ALY) (31). This disease affects *Alnus glutinosa* (L.) Gaertn. (European alder) and *A. incana* (L.) Moench (grey alder) in Europe and *A. rubra* Bong. (*A. oregona* Nutt., red alder) in Washington State (18). Until now, there was no information on the relatedness of the ALY MLO to other MLOs.

In the United States and southeastern Canada, several ash (*Fraxinus*) species are affected by ash yellows (ASHY) (34), which has also been attributed to MLOs (14). The MLOs causing ASHY seem not to be closely related to the EY agent (9,13), although similar symptoms are induced by the two pathogens in the experimental host periwinkle.

The progress made in recent years by the use of molecular methods in characterizing MLOs associated with woody plants is not satisfactory, because most investigations were restricted to relatively few strains that have been transmitted to periwinkle or herbaceous hosts (2,8,9,13,19,21,22). Therefore, little is known about the identity, host specificity, variability, and genetic interrelatedness of such MLOs, especially those which are not available in periwinkle or a herbaceous host. This lack of information is not only a major problem in the understanding, management, and diagnosis of these diseases, it also has an impact on quarantine regulations. In this paper we report on Southern blot analyses in which DNA samples from MLO-infected elm, alder, and ash trees located in Europe and North America were compared with each other and with several MLO strains maintained in periwinkle. An abstract of this work has been published (26).

MATERIALS AND METHODS

Sources of diseased and healthy plants. Samples from EY-diseased American and Chinese (*U. parvifolia* Jacq.) elms were collected in Ithaca, New York, and from an American elm in East Lansing, Michigan. Samples from elm witches'-broom-diseased European field elms were collected in southern France (Avignon) and northern Italy near Trento and Bologna. A Scots elm (*U. glabra* Huds.) was sampled in southwestern Germany near Stuttgart. Samples from 11 ALY-diseased European alder trees were collected in southwestern Germany near Heidelberg and Kaiserslautern, northern Italy near Verona, and Switzerland near Zürich. One diseased *A. incana* tree was sampled in Austria near Innsbruck, and four diseased red alder trees were sampled

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in Washington State near Wenatchee. Samples from nine ASHY-diseased white ash (*F. americana* L.) trees were collected in East Lansing and Ithaca, and near Elyra, Ohio, and Erie, Pennsylvania. Nonsymptomatic field-collected plants from all species examined were used as healthy controls.

The following MLO strains, previously transmitted to periwinkle and maintained in this host by periodic grafting, were included in this study: ASHY1, ash yellows from white ash collected in the northeastern United States; EY1, elm yellows from American elm collected in the northeastern United States; ULW, elm witches'-broom from European field elm obtained from G. Morvan, INRA, Montfavet/Avignon, via F. Dosba, INRA, Bordeaux, France; AT, apple proliferation obtained from R. Marwitz, Biologische Bundesanstalt, Berlin, Germany; ACLR, apricot chlorotic leaf roll obtained from G. Llacer, IVIA, Valencia, Spain, via F. Dosba; MOL, Molières disease of cherry obtained from F. Dosba; PLN, plum leptonecrosis obtained from L. Carraro, University of Udine, Italy; PYLR, peach yellow leaf roll obtained from D. D. Jensen, Berkeley, California, via M. F. Clark, HRI, East Malling, UK; VAC, vaccinium witches'-broom obtained from R. Marwitz; and CVA and PVW, leafhopper-born periwinkle virescence and plantago virescence, respectively, obtained from W. Heintz, Biologische Bundesanstalt, Dossenheim, Germany.

DNA isolation and molecular cloning. MLO DNA from ULW-infected periwinkle plants for molecular cloning was isolated using the cetyltrimethylammonium bromide (CTAB) method and was enriched by repeated bisbenzimide-CsCl buoyant density gradient centrifugation (16). This DNA was digested with *Hin*dIII and ligated into pBluescript SK+ (Stratagene, La Jolla, CA) plasmid DNA. Competent cells of *Escherichia coli*, strain XL1-Blue (Stratagene), were then transformed according to the procedure described by Hanahan (12). Transformants containing recombinant plasmids were identified by plating on Luria-Bertani (LB) agar containing X-Gal (5-bromo-4-chloro-3-indolyl-D-galactoside), IPTG (isopropylthio-D-galactoside), and ampicillin (28). Screening of the transformants for ULW MLO-specific inserts was done by growing white, ampicillin-resistant transformants in liquid LB medium and by isolating the recombinant plasmids using the method of Birnboim and Doly (3). DNA (1.0–2.0 μg) from recombinant plasmids was applied onto a nylon membrane (Hybond N^+, Amersham, Amersham, UK), and the membrane was hybridized as described below (high stringency of the posthybridization washes) with ^{32}P-labeled DNA from healthy and ULW-infected periwinkle plants. To establish whether the cloned ULW MLO DNA fragments were from chromosomal or extrachromosomal MLO DNA, the inserts were used as probes to hybridize Southern blots of undigested DNA from ULW MLO-infected periwinkles. DNA hybridization was carried out as described below employing moderately stringent wash conditions.

Two methods were used to prepare DNA from healthy and diseased plants for Southern hybridization. The MLO-enrichment procedure described by Kirkpatrick et al (15) was modified according to Ahrens and Seemüller (1) to isolate DNA from periwinkle plants. To obtain DNA from elm, alder, and ash trees, midribs of leaves (3.0 g) were cut into small pieces with scissors and ground in liquid nitrogen with a mortar and pestle. The fine powder was extracted according to the protocol of Doyle and Doyle (10). DNA preparations from alder and ash were partially purified by gel filtration on a Sepharose CL-4B (Pharmacia, Uppsala, Sweden) column (20 × 0.6 cm) followed by isopropanol precipitation. Elutip-d columns (Schleicher & Schuell, Dassel, Germany) were used to remove polysaccharides from elm DNA samples.

Preparation of DNA probes and Southern blot analysis. Inserts of recombinant plasmids that hybridized with DNA from ULW MLO-infected plants but not with DNA from healthy plants were excised by *Hin*dIII digestion and were purified by electrophoresis in 1.0% agarose gels. After extraction from the agarose gel by the GeneClean (Bio 101, La Jolla, CA) procedure, the inserts were labeled with ^{32}P dATP with the Multiprime labeling kit (Amersham).

For Southern blot hybridization, approximately 3 μg of sample DNA was digested with *Hin*dIII or *Eco*RI and separated by electrophoresis in horizontal, 1.0% agarose gels by TAE buffer (40 mM Tris-acetate and 1 mM EDTA, pH 8.0) at 3 V/cm. After depurination for 25 min with 0.25 M HCl followed by alkali denaturation, the DNA was transferred to a nylon membrane (Hybond N^+) following the manufacturer's instructions (Amersham). The blots were prehybridized in solutions containing 6× SSC (1× SSC = 0.15 M NaCl and 0.015 M sodium citrate, pH 7.0), 0.5% sodium dodecyl sulfate (SDS), 5× Denhardt's solution (0.1% Ficoll, 0.1% polyvinylpyrrolidone [PVP], and 0.1% bovine serum albumin [BSA]), and 100 μg/ml of salmon sperm DNA at 55 C for 2 h. Hybridization was performed for 16 h at 55 C in the presence of 6× SSC, 0.5% SDS, and 100 μg/ml of salmon sperm DNA. The radiolabeled DNA probe consisted of three different chromosomal fragments (see below) of the ULW MLO. After hybridization, the membranes were washed twice in 2× SSC and 0.1% SDS at room temperature for 15 min, followed by two washes with 0.2× SSC and 0.1% SDS at 55 C (moderate stringency) or at 68 C (high stringency) for 45 min. The membranes were exposed to an x-ray film (Fuji RX, Fuji Photo Film, Düsseldorf, Germany) at −80 C using intensifier screens.

RESULTS

Probe selection. Three recombinant plasmids, which hybridized with DNA from ULW-infected periwinkle but not with DNA from healthy periwinkle, were used as hybridization probes in this study. The sizes of the cloned inserts ULW13, ULW21, and ULW23 excised from these three plasmids were approximately 0.8, 1.7, and 3.5 kb, respectively. When Southern blots of undigested DNA from ULW-infected periwinkle were probed with these fragments, only chromosomal DNA hybridized, and not the smaller DNAs typical for MLO extrachromosomal DNA (*data not shown*). In most Southern hybridizations described below, the three probes were used in equal molar amounts as a mixture.

Probe specificity. The specificity of the three ULW inserts ULW13, ULW21, and ULW23 was determined by using them to probe Southern blots of DNA from periwinkle plants infected by the homologous organism or by 10 other MLOs (Fig. 1). Under moderately stringent wash conditions, the probes hybridized not only to homologous fragments from the ULW MLO but also to identical-size fragments of EY1 MLO DNA. Some of the probes also hybridized with DNA fragments of the ASHY1, VAC, ACLR, and PYLR MLOs. However, the sizes of these fragments from these four MLOs differed from those of the probes. Hybridization signals were absent or very faint between the ULW probes and DNA from the AT, PVW, CVA, PLN, and MOL MLOs.

Southern hybridization of DNA from elm, alder, and ash trees. When *Hin*dIII-digested DNA samples from EY-affected American and Chinese elm trees collected in northeastern United States, and from elm witches'-broom-affected European field elm and Scots elm trees collected in France, Germany, and Italy, were hybridized under moderately stringent conditions with a mixture of the three ULW probes, a very similar pattern was observed in all cases. This pattern was similar to that obtained with strains ULW and EY1 maintained in periwinkle (Fig. 2). A similar hybridization pattern was obtained when DNA samples from 11 ALY MLO-infected European alder trees collected in southwestern Germany, Italy, and Switzerland and one from grey alder collected in Austria were hybridized with these probes (Fig. 3A). Only in the DNA from one German alder tree was an additional fragment, 2.4 kb in size, observed. In all elm samples and in all samples from alder collected in Europe, the probes hybridized to *Hin*dIII-digested DNA fragments that were of the same sizes as the probes. However, only probe ULW13 hybridized strongly with a *Hin*dIII fragment similar to its own size in all four samples of red alder collected in Washington State. The other two probes either hybridized strongly (ULW23) or weakly (ULW21) with only two of the four red alder DNA samples. One of the probes hybridized to a fragment approximately 2.8 kb in size which was present in all samples from red alder but did not occur in samples

from elm or the alders collected in Europe. Additional weak bands were observed when large amounts of DNA from strains EY1, ULW, and some red alders were hybridized with the three probes (Fig. 3B).

The mixture of three ULW probes hybridized with either two or three *Hin*dIII fragments of DNA in each of nine samples from ASHY-affected trees in the northeastern United States (Fig. 4). However, tests with single probes revealed that only probes ULW21 and ULW23 hybridized to DNA of the ASHY MLOs, while probe ULW13 did not. The fragments detected by the two probes were of four sizes, all different from the sizes of the probes (Fig. 4). Also, restriction fragment length polymorphisms (RFLPs) were observed between the ash samples. The RFLP profile of the ash samples collected in Michigan was different from that of the samples collected in New York, Ohio, and Pennsylvania. Both profiles were different from that of the periwinkle-maintained ASHY1 MLO depicted in Figure 1, which shows, in addition to the patterns of the samples collected in New York, Ohio, and Pennsylvania, a fragment of approximately 6 kb in size. A similar-sized fragment was observed when large amounts of DNA from strain ULW and some samples from red alder were hybridized with the three probes (Fig. 3B). None of the probes hybridized to DNA from healthy trees of the elm, alder, and ash species examined (Figs. 2, 3A and B, and 4).

When DNA from ULW-, EY1-, and ASHY1-infected periwinkle plants and a diseased European elm collected in Italy was digested with *Eco*RI and hybridized with the mixture of three ULW probes, the three elm sources showed an identical pattern, while that of ASHY1 MLO was different (*data not shown*).

DISCUSSION

The identities of the MLOs associated with North American elm yellows and European elm witches'-broom were poorly understood. Lethal EY characterized by phloem necrosis was restricted to North America (25,32), and natural infection of European species by North American EY MLOs was unreported. Therefore, the possibility existed that these MLOs differed in host specificity or virulence from those infecting elms in Europe. Thus, the importation of living elm material into European and Mediterranean countries from North America is prohibited by national and European quarantine regulations to prevent introduction of the EY agent (33).

To identify relationships between MLOs in elm and other woody plants, especially alder and ash, and to test relatedness of European and American elm-inhabiting MLOs, Southern hybridization and RFLP analyses were used in this work. These methods have been employed by others to differentiate and group MLOs on a genetic basis (17,20,22). We obtained similar hybridization patterns when Southern blots of DNA samples from two EY-affected elm species grown in Michigan and New York states, and from two elm witches'-broom-affected elm species grown in France, Germany, or Italy were hybridized with three cloned chromosomal DNA fragments of the ULW MLO. These fragments either did not

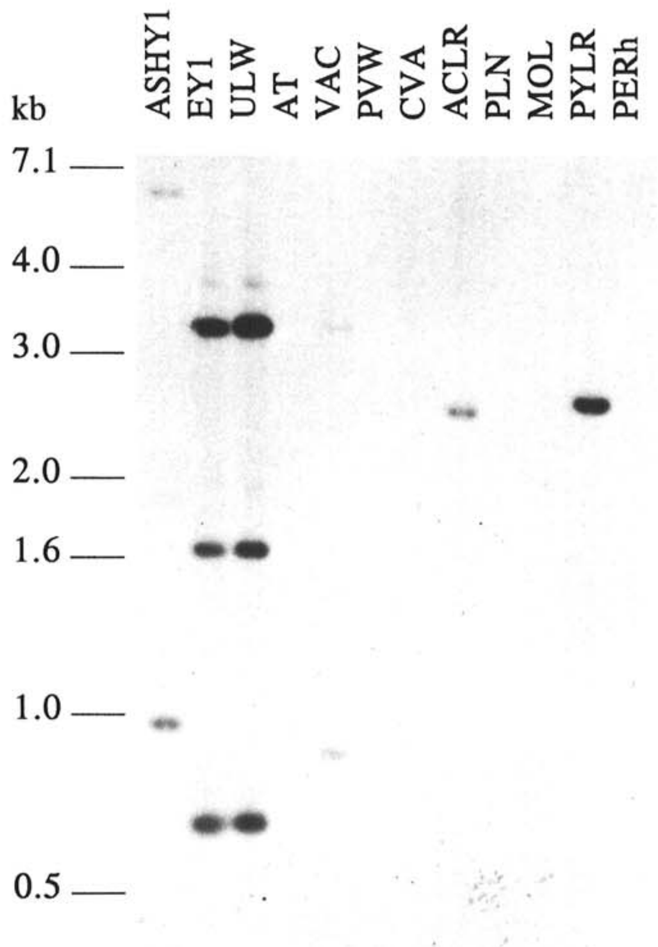

Fig. 1. Southern blot hybridization of a mixture of three cloned DNA probes derived from the European elm witches'-broom mycoplasmalike organism (ULW MLO) to *Hin*dIII-digested DNA from MLO-infected or healthy periwinkle plants. MLO abbreviations: ASHY1, ash yellows; EY1, elm yellows; ULW, European elm witches'-broom; AT, apple proliferation; VAC, vaccinium witches'-broom; PVW, plantago virescence; CVA, catharanthus virescence; ACLR, apricot chlorotic leaf roll; PLN, plum leptonecrosis; MOL, Molières disease; PYLR, peach yellow leaf roll; and PERh, healthy periwinkle.

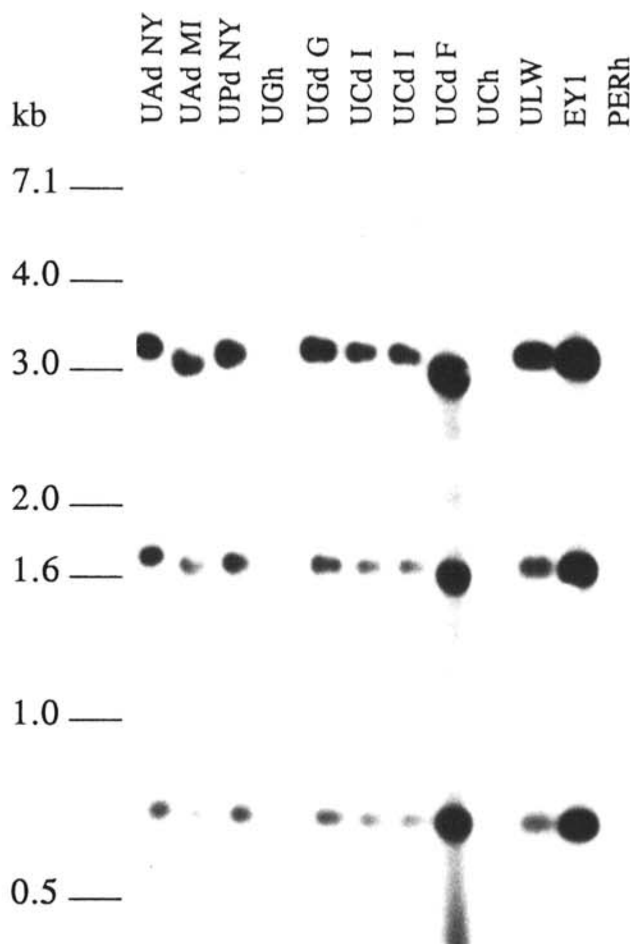

Fig. 2. Southern blot hybridization of a mixture of three cloned DNA probes derived from the European elm witches'-broom mycoplasmalike organism (ULW MLO) to *Hin*dIII-digested DNA from healthy (h) and MLO-diseased (d) elm trees and periwinkle plants. UA, *Ulmus americana*; UP, *U. parvifolia*; UG, *U. glabra*; UC, *U. minor*; EY1, elm yellows; and PER, periwinkle. The tree samples were collected in New York State (NY), Michigan (MI), Germany (G), Italy (I), and France (F).

hybridize with DNA from several other MLOs tested, or hybridized to fragments that were a different size than the probes. Thus, all elm-inhabiting MLOs tested, as well as EY1 and ULW MLOs in periwinkle, have three portions of chromosomal DNA in common that are not shared with any other MLOs tested except those from alder. Therefore, the elm and alder MLOs are closely related to one another and distinct from all others to which they have been compared genetically. Previous workers concerned with MLO identification and classification have noted that elm MLO isolate EY1 in periwinkle differs from periwinkle-maintained MLOs from other hosts (2,8,17,19,21,22). Our finding of close relatedness of European and American elm-infecting MLOs is in agreement with recent results of Lee et al (23), who established the similarity of New York and Italian elm MLOs through the use of MLO-specific DNA probes derived from strain EY1.

There are other findings which support the similarity of the EY and the elm witches'-broom agents and their distant relatedness to other MLOs. Strong evidence was obtained by analyzing the 16S rRNA gene, which is highly conserved among the prokaryotes and for that reason plays an important role in the taxonomy and phylogeny of the mycoplasmas, including the MLOs. Using 16S rDNA that was amplified by a polymerase chain reaction (PCR), Schneider et al (29) observed an identical restriction profile when the 16S rRNA gene of the EY1 and ULW MLOs was digested with the frequently cutting enzymes *Alu*I and *Rsa*I. Schneider et al (29) determined that these two strains, together with the rubus stunt MLO, form a group in which the 16S rDNA restriction profile differs from those of 46 other MLOs examined. Another indication of relatedness of the MLOs that cause EY and elm witches'-broom is that the EY1 and ULW MLOs induce identical symptoms in the experimental host periwinkle (*unpublished observation*).

Elm yellows symptoms in North America vary among elm species. In naturally infected trees, the disease causes chlorosis, defoliation, and death in American elm, and chlorosis, brooming, defoliation, and death in red elm (5,25). Naturally occurring EY has been detected only once, as far as we know, in an Asiatic elm species and never in a European elm species in North America. The single known instance in an Asiatic elm is the diseased *U. parvifolia* examined in this study. Brooming and stunting are also the typical symptoms of European elm witches'-broom, including Italian elm yellows, in naturally infected European field elms (4,6,27) and Scots elm (30), and were also observed after graft inoculation of European field elm with EY (5). That EY and elm witches'-broom are associated with the same symptoms in *U. minor* is another indication of their etiological similarity.

Southern blot hybridization of DNA from ALY-affected alder trees (*A. glutinosa* and *A. incana*) with the three ULW probes resulted in a hybridization pattern very similar to that of DNA from the EY1 and ULW MLOs and from MLO-infected elm trees. Therefore, it can be concluded that not only are EY and elm witches'-broom caused by closely related MLOs, but very similar organisms also affect alder in Europe. Since EY was described (as phloem necrosis [35]) before elm witches'-broom (11), the most appropriate name for the pathogen is elm yellows (EY) MLO.

Although the elm MLO and the MLO from alder collected in Europe were similar in all comparisons made, finer differences among the material, such as host specificity, may not have been detected with the characterization methods employed. Such

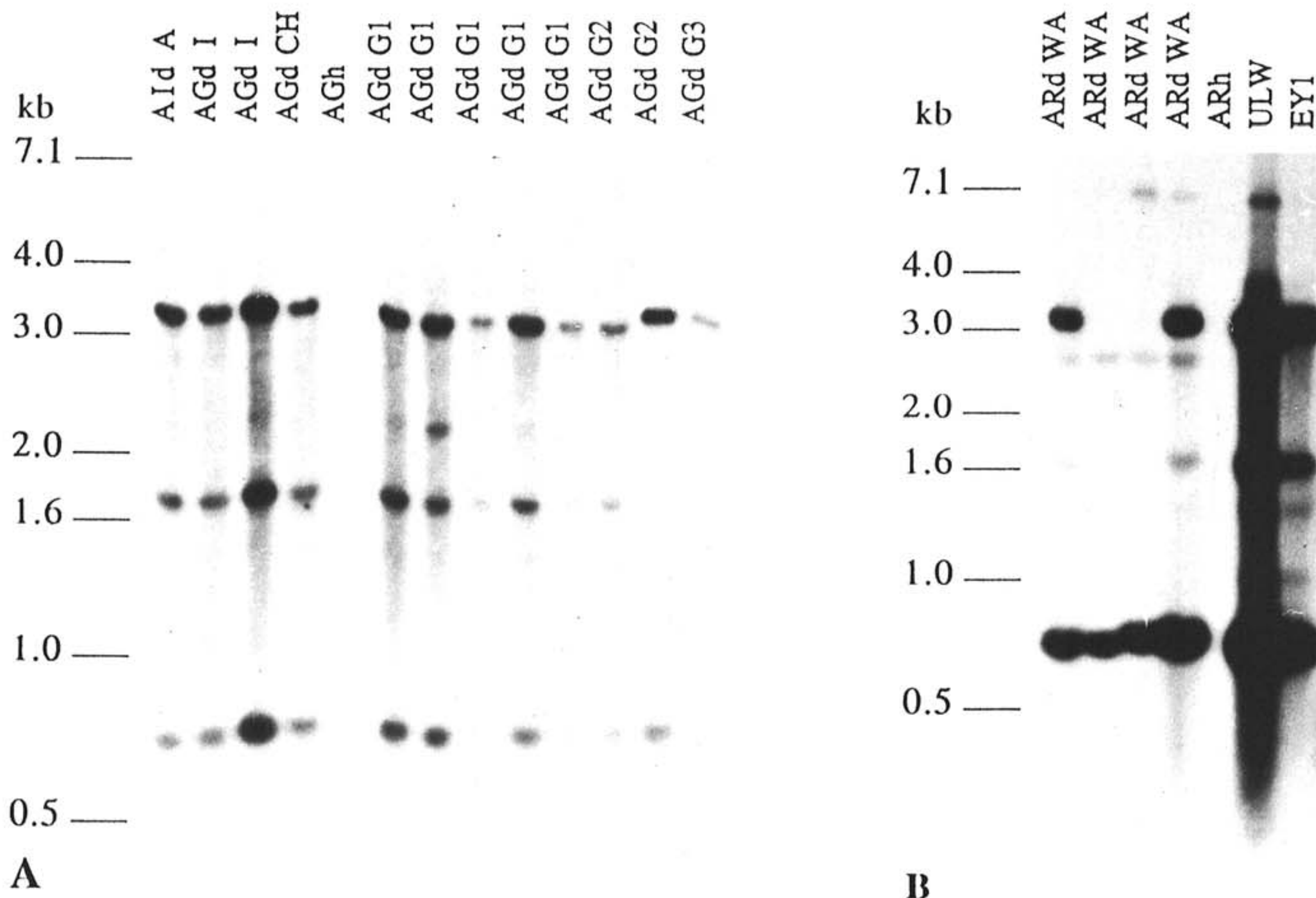

Fig. 3. Southern blot hybridization of a mixture of three cloned DNA probes derived from the European elm witches'-broom mycoplasmalike organism (ULW MLO) to *Hin*dIII-digested DNA from healthy (h) and MLO-diseased (d) trees of *Alnus glutinosa* (AG), *A. incana* (AI), and *A. rubra* (AR) or ULW- and elm yellows (EY1)-infected periwinkle plants. The samples were collected in **A,** Austria (A), Italy (I), Switzerland (CH), and three locations in southwestern Germany (G1–3); or **B,** Washington State (WA). Application of large amounts of DNA resulted in additional (weak) bands in the lanes of the ULW and EY MLOs which are not present in Figs. 1 and 2.

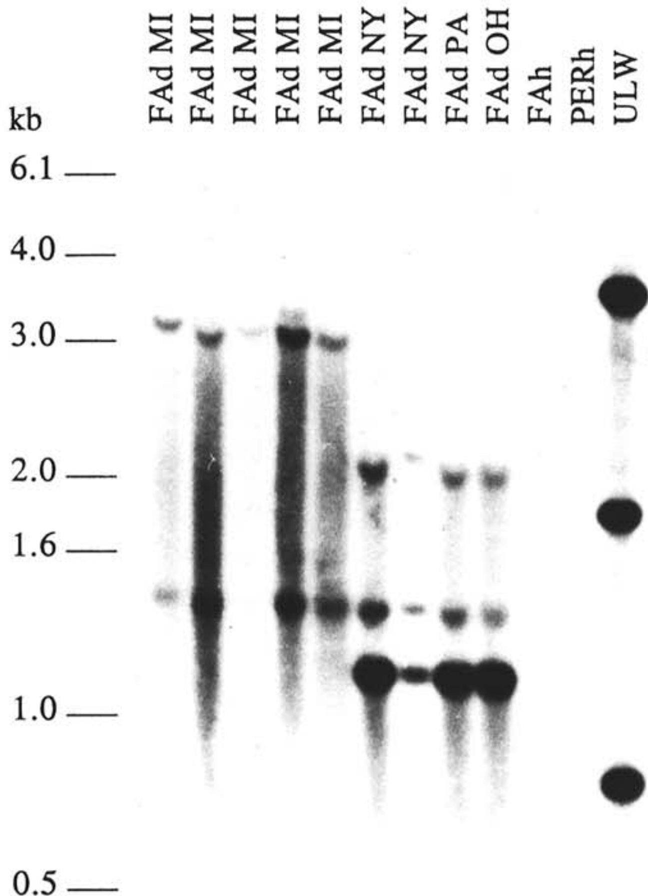

Fig. 4. Southern blot hybridization of a mixture of three cloned DNA probes derived from the European elm witches'-broom mycoplasmalike organism (ULW MLO) to *Hin*dIII-digested DNA from healthy (h) and MLO-diseased (d) trees of *Fraxinus americana* (FA) collected in Michigan (MI), New York State (NY), Pennsylvania (PA), and Ohio (OH), and from healthy (PERh) and ULW-infected periwinkle plants.

differences may be discovered with more specific probes, if they exist, or by serological methods. Also, cross-inoculation experiments might be necessary to demonstrate differences in host specificity. Hybridization patterns different from those of infected elms and alders sampled in Europe were found in the samples from *A. rubra* from Washington State. This finding may indicate either a variability of the MLOs affecting elm and alder or the occurrence of a different MLO in diseased Washington alders.

Two of the three ULW probes hybridized with two or three fragments of all samples from diseased white ash. Therefore, the ASHY agent is more closely related to the MLOs occurring in elm and alder than to all other MLOs that were included in this comparison. However, the elm and alder MLOs belong to a different cluster than do the ash MLOs. This was shown not only in RFLP analyses of the 16S rRNA genes by Schneider et al (29), but also in nucleic acid hybridization studies of Davis et al (9) and Hibben et al (13) in which chromosomal probes from MLO isolate ASHY1 did not hybridize to DNA from isolate EY1 under moderately stringent conditions.

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