Detection and Characterization of an Elm Yellows (16SrV) Group Phytoplasma Infecting Virginia Creeper Plants in Southern Florida

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ABSTRACT

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The polymerase chain reaction (PCR) employing phytoplasma-specific ribosomal RNA primer pair P1/P7 consistently amplified a product of expected size (1.8 kb) from 29 of 36 symptomless Virginia creeper (*Parthenocissus quinquefolia*) plants growing in southern Florida. Restriction fragment length polymorphism analysis of P1/P7-primed PCR products indicated that most phytoplasmas detected in Virginia creeper were similar to phytoplasmas composing the elm yellows (16SrV) group. This relationship was verified by reamplification of P1/P7 products using an elm yellows (EY) group-specific rRNA primer pair fB1/rULWS1. rDNA products (1,571 bp) were generated by group-specific PCR from 28 phytoplasma-positive plants and 1 negatively testing plant identified by earlier P1/P7-primed PCR. Analysis of 16S rDNA sequences determined the Virginia creeper (VC) phytoplasma to be phylogenetically closest to the European alder yellows (ALY) agent, an established 16SrV-C subgroup strain. However, presence or absence of restriction sites for endonucleases *AluI*, *BfaI*, *MspI*, *RsaI*, and *TaqI* in the 16S rRNA and 16-23S rRNA intergenic spacer region of the VC phytoplasma collectively differentiated this strain from ALY and other 16SrV group phytoplasmas. Failure to detect the VC phytoplasma by PCR employing nonribosomal primer pair FD9f/FD9r suggests that this newly characterized agent varies from known European grapevine yellows (flavescence dorée) phytoplasmas previously classified as 16SrV subgroup C or D strains.

Additional keywords: phytoplasma identification, *Vitaceae*

Virginia creeper (*Parthenocissus quinquefolia* (L.) Planch.), also known by other names such as woodbine, American ivy, and five-fingered ivy, is a hardy, fast-growing, deciduous vine native to eastern North America. Wide-ranging in its distribution, this member of the grape family (*Vitaceae*) commonly can be found growing in woodland and hedgerows from Nova Scotia, Canada to southern Florida and as far west as Texas and Oklahoma, where it is often the most prevalent plant species in both bottomland and oak-hickory forests (28).

Virginia creeper (VC) is a close relative of grapevine (*Vitis vinifera* L.), a well-known host of debilitating yellows-type diseases which reportedly occur in many regions of the world where grape is cultivated (1,4,25,29,31).

Etiological studies have implicated the involvement of several distinct phytoplasma strains which differ in their global distribution and incidence in vineyards. Using polymerase chain reaction (PCR) and restriction fragment length polymorphism (RFLP) analysis, strains involved have been shown to belong to groups 16SrI (aster yellows and related strains), 16SrIII (X-disease and related strains), 16SrV (elm yellows and related strains), and 16SrXII (stolbur and related strains) according to the classification system of Lee et al. (20). Distribution of group 16SrV phytoplasmas associated with grapevine flavescence dorée (FD) is apparently limited to viticultural areas of western Europe (2,25,31), although strains serologically related to FD were detected in grapevine and the FD vector leafhopper *Scaphoideus titanus* Ball in New York state (22). Elsewhere in the eastern United States, where grapevine yellows diseases are known to occur, affected *V. vinifera* plants were shown to contain phytoplasmas belonging to groups 16SrI or 16SrIII (4). Symptomless wild grapevine (*V. riparia* Michx.) adjacent to vineyards also harbored phytoplasmas (1,4) capable of infecting cultivated grapevine (22).

Symptoms suggestive of yellows disease were observed on several mature VC vines

growing along fence lines at the University of Florida's Fort Lauderdale Research and Education Center (FLREC) during May 1998. The vines possessed small, crinkled, mildly chlorotic leaves and shoot proliferation which prompted us to examine them for evidence of phytoplasma infection. Although it was learned later that "symptoms" on the plants were a result of herbicide treatment rather than disease, phytoplasmas were detected by PCR in both "symptomatic" plants and "asymptomatic" plants alike during this preliminary investigation (N. A. Harrison, unpublished data). The present study was undertaken during June and July 2000 to determine the identity and incidence of phytoplasmas in this newly recognized vitaceous host species. We report the presence of elm yellows group (16SrV) phytoplasmas in the majority of symptomless VC plants examined at random from among the resident population on the grounds of the FLREC. Detected phytoplasmas were distinct from known grapevine-infecting 16SrV group phytoplasmas, and phylogenetically closest to European alder yellows (ALY) agent (26), previously designated as a 16SrV-C subgroup member (20).

MATERIALS AND METHODS

Plant samples and phytoplasma reference strains. Samples consisting of young leaves from the tips of vines were collected during May 1998 and in June and July 2000 from a total of 36 apparently symptomless VC plants on the grounds of the FLREC. Phytoplasmas used for comparative purposes included the following strains: elm yellows (EY1) (7), eastern aster yellows (EAY), western X (WX), Mexican periwinkle virescence (MPV) (11), and pigeonpea witches'-broom (PPWB) (14), each maintained separately in periwinkle (*Catharanthus roseus* (L.) G. Don). A Florida strain of the maize bushy stunt (MBSF) phytoplasma was maintained in sweet corn (*Zea mays* L. var. *saccharata* (Sturtev.) L. H. Bailey) by periodic transmissions using vector *Dalbulus maidis* leafhoppers (13). DNA of the palm lethal yellowing (LY) phytoplasma was obtained from immature leaf tissues of a coconut palm (*Cocos nucifera* L.) displaying typical LY symptoms (12). The beet leafhopper-transmitted virescence agent (BLTVA), Brachiara white leaf (BraWL), flavescence dorée (FD70), Gliricidia (*G. sepium*) little

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leaf (GLL), jujube (*Ziziphus jujube*) witches'-broom (JWB), sweet potato (*Ipomoea batatas*) little leaf (SPLL), and *Rubus* stunt (RuS) strains were provided to us by other researchers either as DNA samples or in plant tissues. Seedling periwinkle was used as a healthy standard because known healthy VC plants were unavailable during this study.

DNA extraction. Total nucleic acid was extracted from 0.5 g of dried or 3 g of fresh plant foliage by the method of Doyle and Doyle (6), except for VC tissues, which were extracted using a modified phytoplasma enrichment method (17) as follows. Samples (1.5 g) of freshly harvested leaf petioles were diced with a razor blade and ground thoroughly in a mortar and pestle containing 12 ml of warm (25°C) grinding buffer and sea-washed sand to facilitate the grinding process. The resulting viscous slurry was transferred to a sterile 30-ml Corex centrifuge tube and diluted with an additional 15 ml of grinding buffer before centrifugation at 3,000 × g for 10 min at 25°C. The partially clarified supernatant was decanted to another sterile 30-ml Corex tube and centrifuged again at 20,000 × g for 30 min. After discarding the supernatant, nucleic acids were extracted from the residual pellet as previously stated (6). Ethanol-precipitated nucleic acids derived from each pellet were resuspended in 50 to 100 µl of TE (10 mM Tris, 1 mM EDTA, pH 8) buffer containing RNase and incubated at 37°C for 1 h. Resulting DNA extracts were quantified by fluorometry (TK-100 minifluorometer; Hoefer Scientific, San Francisco, CA) and stored at 4°C before use.

PCR analysis. DNA samples used as templates for PCR were first diluted to 25 ng/µl with sterile deionized water. Amplifications were performed in 50-µl final reaction volumes, each containing 50 ng of DNA template, 50 ng of each primer, 125 µM of each dNTP, 1 U of Taq DNA polymerase (Promega Corp., Madison, WI), and standard PCR buffer containing 1.5 mM MgCl₂ (15). PCR was performed for 35 cycles in a PTC/100 programmable thermal controller with hot bonnet (MJ Research Inc., Watertown, MA) employing phytoplasma-specific rRNA primer pair P1 and P7, which amplifies almost the entire 16S rRNA gene, the 16-23S rRNA spacer region, and the 5'-end of the 23S rRNA gene (38). The following thermal cycling parameters were used: denaturation for 30 s (90 s for first cycle) at 94°C, annealing for 50 s at 55°C, and extension for 80 s at 72°C. Reactions were terminated after the final cycle with a 10-min extension step at 72°C, then cooled to 4°C. VC DNAs were subsequently reassessed by PCR employing nonribosomal primers FD9f/FD9r using previously reported thermal cycling conditions (2).

Products resulting from P1/P7 amplifications were diluted 1:40 or 1:100 with sterile deionized water and 3 µl of each dilution, then used as template during 30 cycles of PCR with nested elm yellows group-specific rRNA primer pair fB1/rULWS1, as previously described (38). Aliquots (5 or 10 µl) of each PCR reaction were evaluated by electrophoresis through 1% agarose (low EEO; Fisher Scientific, Pittsburgh, PA) gels using 1×

TAE (40 mM Tris-acetate, 1 mM EDTA) as running buffer. Products in gels were stained with ethidium bromide (EtBr), visualized by UV transillumination, and photographed.

DAPI staining test. Serial transverse sections were cut with a razor blade from petioles of VC plants known to contain phytoplasmas following their assessment by PCR. Sections were stained with an aqueous solution of the DNA-binding fluorochrome DAPI (4'-6-diamidino-2-phenylindole), mounted in clear corn syrup on a microscope slide, and examined for phytoplasma infection by epifluorescence microscopy as described by Sinclair et al. (37).

RFLP analysis of PCR products. Aliquots (5 µl) of each P1/P7-primed PCR product were digested separately with 10 U of restriction endonucleases *Bfa*I (New England BioLabs, Beverly, MA), *Alu*I, *Dde*I, *Dra*I, *Eco*RI, *Hae*III, *Hha*I, *Msp*I, *Rsa*I, and *Sau*3AI (Promega Corp.) at 37°C, with *Bst*UI (New England BioLabs) at 60°C, or with *Taq*I and *Tru*9I (Promega Corp.) at 65°C for a minimum of 16 h. Digests were separated by electrophoresis through 8% nondenaturing polyacrylamide gels using 1× TBE (90 mM Tris-borate, 2 mM EDTA) as running buffer. Products in gels were resolved and recorded as previously described.

Sequencing of PCR products. P1/P7-primed rDNA products from VC sample VC2 and jujube witches'-broom (JWB) were each purified on spin columns (QIAquick PCR Purification Kit; Qiagen, Valencia, CA) and eluted with sterile ul-

Table 1. Description of phytoplasma 16S rDNA sequences used in this study

Phytoplasma or associated disease	16S rRNA group-subgroup affiliation	GenBank accession no.	Reference
Michigan aster yellows (MiAY)	16SrI-B	M30790	21
Japanese hydrangea phyllody (JHP) ("Candidatus Phytoplasma japonicum")	16SrI-D	AB010425	32
Peanut witches'-broom (PnWB)	16SrII-A	L33765	10
Witches'-broom disease of lime (WBDL) ("Candidatus Phytoplasma aurantifolia")	16SrII-B	X76433	42
Faba bean phyllody (FBP)	16SrII-C	X83432	34
Western X (WX)	16SrIII-A	L04682	34
Clover yellow edge (CYE)	16SrIII-B	L33766	10
Palm lethal yellowing (LY)	16SrIV-A	U18747	39
Elm yellows (EY1)	16SrV-A	AF189214	7
Elm yellows (EY-WV)	16SrV-A	AF122911	7
Ulmus witches'-broom (ULW)	16SrV-A	X68376	34
Jujube witches'-broom (JWB)	16SrV-B	AF305240	This article
Alder yellows (ALY)	16SrV-C	Y16387	34
Hemp dogbane yellows (HD1)	16SrV-C	AF122912	7
Virginia creeper (VC)	16SrV-C	AF305198	This article
Flavescence dorée (FD)	16SrV-D	X76560	34
<i>Rubus</i> stunt (RuS)	16SrV-E	Y16395	34
Clover proliferation (CP)	16SrVI-A	L33761	10
Ash yellows (AshY1) ("Candidatus Phytoplasma fraxini")	16SrVII-A	AF189215	9
Loofah witches'-broom (LfWB)	16SrVIII-A	L33764	10
Pigeon pea witches'-broom (PPWB)	16SrIX-A	U18763	39
Apple proliferation (AT)	16SrX-A	X68375	34
Buckthorn witches'-broom (BWB)	16SrX-E	X76431	34
Rice yellow dwarf (RYD)	16SrXI-A	D12581	27
Stolbur (STOL)	16SrXII-A	X76427	34
Australian grapevine yellows (AUGY) ("Candidatus Phytoplasma australiense")	16SrXII-B	X95706	29
Bermuda grass white leaf (BGWL)	16SrXIV	Y16388	34
Hibiscus witches'-broom (HibWB) ("Candidatus Phytoplasma brasiliense")	16SrXV	AF147708	See addendum
<i>Acholeplasma laidlawii</i>	Not applicable	M23932	41

trapure water. The VC phytoplasma rDNA product was then sequenced directly using automated equipment at Cornell University's Biotechnology Center. The JWB rDNA product was cloned in vector pGEM-T (Promega Corp.) and *Escherichia coli* DH5 α cells (BRL Life Technologies, Rockville, MD) according to the manufacturer's instructions prior to automated sequencing at the University of Florida's Core DNA sequencing laboratory. The following primers were used to obtain extended sequence reads from the cloned JWB rDNA product: P1, P7, (38), 503f (5'-CAGCAGCCGCGGTAATACATA-3'), 503r (5'-TATGTATTACCGCGGCTGCTG-3'), 1033f (5'-TTGTCGTCAGCTCGTGTC-3'), and 1033r (5'-GACACGAGCTGACGACAA-3').

Analysis of phytoplasma rDNA products. Sequences derived from VC and JWB phytoplasma P1/P7-primed rDNA products were assembled and putative endonuclease recognition sites in each sequence were mapped using Vector NTI 5 Suite software (Informax Inc., Bethesda, MD). Maps were examined for concordance with restriction sites previously identified by enzymatic digestion and compared with rDNA sequences of related phytoplasmas selected from the GenBank database. Pairwise comparisons of phytoplasma 16S rDNA sequences were obtained by Gap analysis (Wisconsin package version 10.1; Genetics Computer Group [GCG], Madison, WI). For phylogenetic analysis, 16S rDNA sequences of the VC and JWB phytoplasmas, 26 other phytoplasma strains representing 14 primary phytoplasma groups according to the classification scheme of Lee et al. (20), and *Acholeplasma laidlawii* (Table 1) were edited and aligned using SeqEd and PileUp

programs (Wisconsin package version 10.1). Pairwise evolutionary distances between aligned sequences were created by Distances (SeqWeb version 1.2; GCG) incorporating the Kimura-2 parameter distance correction method. A phylogenetic tree was reconstructed by Neighbor-Joining from the distance matrix using GrowTree (SeqWeb) and visualized by TreeView (30).

RESULTS

Phytoplasma detection in VC. Template DNA from VC samples suitable for PCR was readily obtained with minor modifications to the phytoplasma enrichment method. Phytoplasmas were detected by P1/P7-primed PCR in 29 of 36 (80.5%) symptomless VC plants sampled on the grounds of the FLREC during May 1998 and June and July 2000. Positive detections were identified as weak or moderate amplification of an rDNA product of about 1.8 kb. Presence of at least one additional smaller size band in gel profiles indicated that nontarget products were also amplified from some plant samples. Amplification products typical of those obtained by P1/P7-primed PCR are illustrated in Figure 1A.

Phytoplasma infection of VC determined by PCR was also investigated by the DAPI staining test. Fluorescence due to DAPI staining was clearly observed in transverse sections from only two of eight plants examined by this means. In these plants, staining was confined to a few scattered phloem sieve tubes of sections, suggesting that phytoplasmas were present in these tissues in low titer. Unequivocal detection of phytoplasmas in six remaining test plants was prevented by a constitutive

reddish pigment, presumably anthocyanins, which leached from petiole sections and masked sieve tube staining (*data not shown*).

Phytoplasma characterization. When *RsaI* digests of P1/P7 amplification products derived from phytoplasma-infected VC sample VC2 and 12 additional strains representative of nine distinct phylogenetic groups of phytoplasmas were compared, resulting rDNA fragment profiles indicated the phytoplasma associated with this plant was most similar to elm yellows phytoplasma EY1, an established 16SrV group member (Fig. 2). Reamplification of a 1,571-bp product from primary P1/P7 products by PCR incorporating nested rRNA primer pair fB1/rULWS1 demonstrated that 28 additional VC plants also contained EY group phytoplasmas. These included 27 known phytoplasma-positive plants and 1 negatively testing plant based on the outcome of initial P1/P7-primed PCR (Fig. 1B). Only P1/P7-positive sample VC17 failed to yield an rDNA product as a result of this nested PCR assay, indicating that this particular plant harbored a phytoplasma other than an EY group strain. The identity of the latter phytoplasma remains to be determined.

Similarity between VC-inhabiting phytoplasmas and other known group 16SrV strains was substantiated by additional separate digestions of P1/P7 products with

Fig. 1. Agarose gel electrophoresis of rDNA products amplified from DNAs of symptomless Virginia creeper (*Parthenocissus quinquefolia*) plants by polymerase chain reaction (PCR) assays. Products generated by **A**, PCR incorporating phytoplasma-specific rRNA primer pair P1/P7 and **B**, reamplification of P1/P7 products with nested elm yellows (EY) phytoplasma group-specific rRNA primer pair fB1/rULWS1. Template DNA samples were derived from: VC, Virginia creeper plants; HP, healthy periwinkle; EY1, elm yellows phytoplasma; JWB, jujube witches'-broom phytoplasma; and WC, water control. E/H, *HindIII/EcoRI* digested lambda DNA, fragment sizes (kb) in descending order: 21.12, 5.11, 4.97, 4.3, 3.53, 2.03, 1.9, 1.58, 1.37, 0.95, 0.83, 0.56, 0.12.

Fig. 2. Comparison of restriction fragment profiles resulting from *RsaI* endonuclease digestion of rDNA products (1.8 kb) amplified from various phytoplasma-infected plants by a polymerase chain reaction (PCR) assay incorporating rRNA primer pair P1/P7. Fragment profiles were resolved by ethidium bromide staining after electrophoresis through 8% polyacrylamide gels. PCR products were derived from the following phytoplasma DNA templates: VC2, symptomless Virginia creeper; EY1, elm yellows; EAY, eastern aster yellows; MPV, Mexican periwinkle virescence; BLTVA, beet leafhopper-transmitted virescence agent; WX, western X; PPWB, pigeon pea witches'-broom; JWB, jujube witches'-broom; MBSE, maize bushy stunt; SPL, sweet potato little leaf; coconut lethal yellowing; GLL, Gliricidia little leaf; and BraWL, Brachiaria white leaf. pGEM: molecular size (bp) markers in descending order: 2645, 1605, 1198, 676, 517, 460, 396, 350, 222, 179, 126, 75, 65, 51, 36.

AluI, *BfaI*, *MspI*, *RsaI*, or *TaqI*, because these endonucleases collectively produced the most informative rDNA RFLP patterns during an earlier analysis of phytoplasma strain VC2 employing a total of 13 endonucleases (*data not shown*). Digests with *AluI* revealed no clear differences between VC phytoplasmas and strains EY1, JWB, FD70, or RuS (Fig. 3A), whereas *MspI* and *RsaI* digests each distinguished the JWB phytoplasma from the other strains (Fig. 3B and C). *BfaI* digests produced seemingly identical fragment profiles for VC phytoplasmas, FD70, and RuS which were distinct from profiles of strains EY1 and JWB (Fig. 3D). *TaqI* profiles distinguished VC phytoplasmas from all other strains except JWB (Fig. 3E), whereas *MspI*, *RsaI*, and *TaqI* profiles collectively differentiated VC phytoplasmas from the other four group-16SrV strains.

For infected VC plants that yielded insufficient P1/P7 product to permit RFLP analysis, the identity and uniformity of the associated phytoplasmas was determined

by *BfaI*, *RsaI*, or *TaqI* (Fig. 4) digestion of nested products obtained by fB1/rULWS1-primed PCR. Fragment profiles revealed by these analyses were uniform and conformed to those obtained for strain VC2, indicating that each of these additional plants also contained very similar or possibly the same phytoplasma strain.

Thirty-five cycles of PCR primed by nonribosomal primer pair FD9f/FD9r amplified a DNA product of anticipated size (1.3 kb) from EY1, FD70, HD1, RuS, and JWB phytoplasma DNAs. By comparison, this assay consistently failed to generate a discernible product from any VC plants. Lack of amplification indicated that phytoplasmas infecting the latter host species varied from the other established 16SrV group strains in another as yet uncharacterized region of their genomes (*data not shown*).

Nucleotide sequence, sequence similarities, and putative restriction sites in phytoplasma rRNA operon sequences. The rDNA sequences of VC and JWB

phytoplasma strains determined in this study have been deposited in the GenBank database under accession numbers AF305198 and AF305240, respectively. Pairwise similarities between 16S rDNA sequences of these strains and those of other known 16SrV phytoplasmas ranged from 98.99 to 99.93%, respectively. This range of values is consistent with the inclusion of both VC and JWB phytoplasmas in the EY phytoplasma group, as was deduced from earlier rDNA RFLP comparisons (this article). Based on putative restriction site analysis, both phytoplasmas could be differentiated from each other and from five other EY group strains by presence or absence of key *AluI*, *BfaI*, *MspI*, *RsaI*, and *TaqI* endonuclease recognition sites in their respective 16S and 16-23S rDNA sequences (Fig. 5). A combination of single sites for *BfaI* and *RsaI* located at positions 1061 and 1243, respectively, in the 16S rDNA, along with an *AluI* site at position 1607 and two sites for *TaqI* at positions 1653 and 1700 in the 16-23S spacer region,

Fig. 3. Restriction fragment length polymorphism (RFLP) profiles of rDNA (1.8 kb), amplified by the polymerase chain reaction (PCR) with primer pair P1/P7 from Virginia creeper (*Parthenocissus quinquefolia*) and other plant species infected with elm yellows (16SrV) group phytoplasmas. Fragment profiles were resolved by ethidium bromide staining after electrophoresis through 8% polyacrylamide gels. Template DNAs for PCR were derived from VC, symptomless Virginia creeper plants; or phytoplasma strains EY1, elm yellows; JWB, jujube witches'-broom; FD70, flavescence dorée; and RuS, Rubus stunt. P1/P7 products were digested with **A**, *AluI*, **B**, *MspI*, **C**, *RsaI*, **D**, *BfaI*, or **E**, *TaqI*. pGEM: DNA molecular size (bp) markers in descending order: 2645, 1605, 1198, 676, 517, 460, 396, 350, 222, 179, 126, 75, 65, 51, 36.

Fig. 4. Comparison of fragment profiles resulting from restriction endonuclease digestion of rDNA products (1.57 kb) reamplified from symptomless Virginia creeper (*Parthenocissus quinquefolia*) plants by a polymerase chain reaction (PCR) assay incorporating nested elm yellows phytoplasma (16SrV) group-specific rRNA primer pair fB1/rULWS1. Template DNAs for PCR were derived from VC, symptomless Virginia creeper plants; EY1, elm yellows phytoplasma; and JWB, jujube witches'-broom phytoplasma, and digested with **A**, *Bfa*I, **B**, *Rsa*I, and **C**, *Taq*I. pGEM: DNA molecular size (bp) markers in descending order: 2645, 1605, 1198, 676, 517, 460, 396, 350, 222, 179, 126, 75, 65, 51, 36.

Fig. 5. Analysis of putative restriction sites in rRNA operon sequences of Virginia creeper-associated phytoplasma strain VC2 and related elm yellows (16SrV) group phytoplasmas. Linear maps were generated using the restriction map option in Vector NTI 5 Suite software (Informax Inc., Bethesda, MD). The region corresponding to the 16-23S rDNA intergenic spacer region sequence is indicated by black color on each map. The identity of each phytoplasma rDNA sequence is as follows: VC, Virginia creeper; ALY, European alder yellows; JWB, jujube witches'-broom; FD88, flavescens dorée; EY1, elm yellows; RuS, Rubus stunt; and HD1, hemp dogbane yellows. Numbers in parentheses in tandem with restriction enzyme labels on maps denote the location of restriction sites within the rDNA sequence. Labels enclosed in boxes delineate restriction site combinations that are uniquely represented in each associated phytoplasma sequence.

was unique for the VC phytoplasma whose rDNA sequence (1,744 bp) varied from that of strain ALY (1,744 bp) at only four positions. Of these, substitution of an A for G at position 1655 in the 16-23S intergenic spacer region of strain ALY eliminated a restriction site for *TaqI* that was present in the VC phytoplasma. The occurrence of an *MspI* site at 16S rDNA position 1242 in conjunction with *TaqI* sites at positions 1651 and 1698, respectively, within the 16-23S spacer, uniquely defined the JWB phytoplasma, a previously designated 16SrV-B subgroup member (20). Phytoplasma strains FD70, EY1, RuS, and HD1 could be similarly differentiated by this means, although the available sequence data for RuS and HD1 was insufficient to establish the existence of a *TaqI* site located at position 15 on the 16S rRNA gene of all five other strains. Putative restriction site analysis also revealed a unique *RsaI* recognition site located at position 733 in the rDNA sequence of the RuS phytoplasma. By comparison, we noted that the fragment profile generated by actual enzymatic digestion of RuS phytoplasma P1/P7 product with *RsaI* also matched the

profiles similarly obtained for strains EY1, FD70, and VC (Fig. 3C). As such, we were unable to confirm the occurrence of an extra *RsaI* site at position 733 in the rDNA sequence of the RuS phytoplasma.

Phylogenetic analysis. A phylogenetic distance tree was constructed from a data set which included 16S rDNA sequences of the VC and JWB phytoplasma strains and comparable sequences of 26 additional strains representing 14 previously established RFLP groups (Fig. 6). The branching orders resolved by this analysis were similar to and supported the same major phylogenetic groups identified in other studies (3,10). In this analysis, both the VC and JWB phytoplasmas clustered closely together along with seven other established EY group members, thereby supporting their assignment to the EY (16SrV) group. Tree branching patterns also indicated that the VC phytoplasma was evolutionarily closest to the European ALY phytoplasma and part of an existing lineage (subgroup 16SrV-C) of strains. By comparison, the JWB phytoplasma gave rise to a new branch not included in previous phylogenetic trees and indicated that this strain

represents a separate lineage (subgroup) within the EY group, a prior assertion drawn from results of rDNA RFLP analysis.

DISCUSSION

The majority of VC plants examined in this study contained phytoplasmas even though none of the infected plants showed any obvious yellows disease symptoms. Similar latent infections have been detected previously in alder (18), velvet ash (36), apricots (16), almonds (40), and wild grapevine (1). These observations support the epidemiological concept of the existence of symptomless host plants which serve as reservoirs for phytoplasma increase and dispersal (35).

Our identification of these strains as members of 16S rDNA RFLP group 16SrV sensu Lee et al. (20) was unexpected because, prior to this finding, the known host range and geographic distribution of 16SrV phytoplasmas in North America was limited to elm (7), hemp dogbane (8), and possibly grapevine (1,22) in the northeastern United States. Within group 16SrV, both elm- and hemp dogbane-infecting

Fig. 6. Phylogenetic tree of 16S rRNA gene sequences from 28 phytoplasmas and *Acholeplasma laidlawii* constructed by the Neighbor-Joining method. 16S rRNA restriction fragment length polymorphism group identities are indicated by Roman numerals.

strains have been further delineated into subgroups 16SrV-A and 16SrV-C, respectively, based on differences in endonuclease recognition sites in their 16S rDNA sequences (2,20). Using this same approach to classification in our work, VC-associated phytoplasmas conformed to previously designated subgroup 16SrV-C members.

In contrast to only limited variability of known 16SrV phytoplasmas in the United States, strains representative of at least four 16SrV subgroups have been implicated as the etiological agents of diseases affecting a broader range of host plant species in Europe. These include strains associated with *Spartium witches'-broom* (SpaWB) (34), little leaf of *Eucalyptus* (24), alder yellows (18), and flavescence dorée of cultivated grapevine, *V. vinifera* (3), all designated members of subgroup 16SrV-C (20). The *Rubus* stunt phytoplasma (19) previously classified as a 16SrV-C strain (20) was recently re-assigned to a new subgroup, 16SrV-E, after RFLP patterns generated with endonuclease *Tsp509I* were shown to distinguish this phytoplasma from others within subgroup 16SrV-C (3). By mapping putative recognition sites for *Tsp509I* in P1/P7-primed rDNA sequences, we found that this endonuclease should be useful for differentiating the VC phytoplasma from other EY group strains, with the exception of ALY, whose sequence yielded a restriction map identical to that of the VC phytoplasma (*data not shown*).

Griffiths et al. (7) showed that molecular variability among elm-inhabiting and other 16SrV phytoplasmas could be defined more precisely by considering disparities in key restriction sites for endonucleases *AluI*, *BfaI*, *HpaII* (= *MspI*), *RsaI*, and *TaqI* in a portion of the rDNA operon that included both the 16S rRNA gene and the noncoding 16-23S rRNA spacer region. By adopting this same approach to characterization of the VC phytoplasma, it was possible to differentiate this agent from other 16SrV phytoplasmas included for comparative purposes. Moreover, presence or absence of putative restriction sites for *AluI* and *TaqI* in the 16-23S spacer region revealed VC, HD1, and ALY as three distinct rather than coincidental 16SrV-C strains, as could have been deduced from putative RFLP analysis of 16S rDNA sequences alone. These findings substantiate and augment results of other studies (3,7) and suggest that subgroup classification based solely on 16S rDNA is probably insufficient to fully account for variability among 16SrV group phytoplasmas. Further support for this contention was also provided by the outcome of PCR with nonribosomal primer pair FD9f/FD9r, an assay that reportedly detects FD (2) as well as other 16SrV group phytoplasmas, such as HD1 (8). In our study, this assay consistently failed to amplify any detectable

product from phytoplasma-infected VC plants. Thus, sequence variation in the 16-23S spacer region between the VC phytoplasma and other 16SrV phytoplasmas was also accompanied by a demonstrable difference in another as yet uncharacterized region of the genome of the former strain.

Analyses of additional conserved genes, such as ribosomal protein (rp) genes (10), and the gene encoding elongation factor *Tu* (33) have been used to support and refine subgroup classifications based upon 16S rRNA sequences. Presently, these efforts have been focused primarily on phytoplasmas composing groups 16SrI (aster yellows and related strains) (23) and 16SrIII (western X and related strains) (11) and, to a much lesser extent, on strains composing group 16SrV. By inclusion of rp gene RFLPs in a revision of an earlier classification scheme, Lee et al. (20) demonstrated that further distinction between the 16SrV-B subgroup phytoplasmas cherry lethal yellows (CLY) and JWB was possible. These results suggest that rp gene analysis could help to clarify relationships between other members of this taxonomic group.

In the absence of known symptoms, plant host range, and vector insects, the phytopathological significance of phytoplasma-infected VC remains to be determined. Further examination of this ecologically dominant species for evidence of phytoplasma infection at other sites in southern Florida and in areas where diseases attributed to 16SrV group phytoplasmas have been previously described seems warranted. The high incidence of infection among plants sampled at the FLREC suggests a close ecological association between this plant species and insects that serve as vectors of the VC phytoplasma. Knowledge of this relationship should facilitate vector identification attempts. Of the currently recognized plant hosts of 16SrV phytoplasmas, several *Rubus* and wild *Vitis* spp. are indigenous to southern Florida. Whether these plants and eucalyptus, an introduced species, represent possible alternative hosts of the VC phytoplasma is unknown. Only the ubiquitous wild grapevines were found growing in close proximity to VC at the FLREC. Thus far, we have not observed yellows disease symptoms on this candidate host, nor have we detected 16SrV group phytoplasmas in symptomless grapevines or in *Ampelopsis arborea* (L.) Koehne (peppervine), another native vitaceous species (N. A. Harrison, unpublished data). Currently, these provisional observations lend support to the hypothesis that diseases attributed to different 16SrV subgroup phytoplasmas may be mutually exclusive (5).

ADDENDUM

After this paper was accepted for publication, the following paper was published: Montano, H. G., Davis, R. E., Dally, E. L., Hogenhout, S., Pimentel, J. P., and Brioso, P. S. T. 2001. '*Candidatus* Phytoplasma brasiliense', a new phyto-

plasma taxon associated with hibiscus witches' broom disease. *Int. J. Syst. Evol. Microbiol.* 51:1109-1118.

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