The phytoplasma associated with ash yellows and lilac witches'-broom: 'Candidatus Phytoplasma fraxini'

Helen M. Griffiths,† Wayne A. Sinclair,† Christine D. Smart† and Robert E. Davis

Phytoplasmas associated with the plant diseases ash yellows (AshY, occurring in Fraxinus) and lilac witches'-broom (LWB, occurring in Syringa) represent a putative species-level taxon. Phytoplasmal DNA from 19 ash or lilac sources across the known geographic range of AshY (71–113°W) was examined to determine if AshY and LWB phytoplasmas are a coherent group, if variability exists in both conserved and anonymous DNA, and if variability in 16S rDNA is related to host or geographic origin. The 16S rRNA gene and the 16S–23S spacer were amplified using primer pair P1/P7 and analysed using 15 restriction enzymes. RFLPs were detected in digests obtained with AluI, Hhal or TaqI, for a total of four RFLP profile types. Sequencing of the amplimers from strains AshY1, AshY3, AshY5 and LWB3 (which represent the four 16S rDNA RFLP profile types) revealed only three positions in the 16S rRNA gene and one position in the 16S–23S spacer at which differences occurred; these were single nucleotide substitutions. Sequence homology between any two strains was >99.8%. A portion of a ribosomal protein operon, amplified with primer pair rpf1/rf1 from each of the four strains noted above, was analysed with six restriction enzymes, resulting in the detection of two RFLP profiles with MseI. Southern analysis, utilizing two non-specific probes from other phytoplasma groups, revealed three RFLP profile types in anonymous chromosomal DNA of strains representing the four 16S rDNA genotypes. Two strains, AshY3 and LWB3, had unique combinations of characters in the various assays. On the basis of RFLP profiles, the strains from the other plants sampled comprised two groups. The grouping was not clearly related to host or geographic origin. The genome size of strain AshY3 was estimated from PFGE data to be 645 kbp.

Phylogenetic analysis of a 1423 bp 16S rDNA sequence from strains AshY1, AshY3, AshY5 and LWB3, together with sequences from 14 other mollicutes archived in GenBank, produced a tree on which the AshY and LWB strains clustered as a discrete group, consistent with previous analyses utilizing only type strain AshY1. Thus, the AshY phytoplasma group is coherent but heterogeneous. The name 'Candidatus Phytoplasma fraxini' is proposed for this group.

Keywords: 16S rRNA, plant disease, Fraxinus, Syringa, 'Candidatus Phytoplasma fraxini'

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Abbreviations: AshY, ash yellows; AY1, Maryland aster yellows; BB, tomato big bud; BLL, brinjal little-leaf; CP, clover proliferation; CX, Canadian peach X-disease; CYE, clover yellow edge; EY1, elm yellows; LFWB, loofah witches'-broom; LWB, lilac witches'-broom; PnWB, peanut witches'-broom; PPWB, pigeon pea witches'-broom; SPWB, sweet potato witches'-broom; ULW, elm yellows.

The GenBank accession numbers for the P1/P7 amplimer (the 16S rDNA gene and the 16S–23S spacer) of phytoplasma strains AshY1, AshY3, AshY5 and LWB3 are AF092209, AF105315 and AF105317, respectively.
INTRODUCTION

Ash yellows (AshY) and lilac witches'-broom (LWB) are diseases of Fraxinus spp. and Syringa spp. (Oleaceae) putatively caused by phytoplasmas (Sinclair & Griffiths, 1994; Sinclair et al., 1996). Phytoplasmas are non-culturable pleomorphic mollicutes that inhabit plant phloem and insects, mainly leafhoppers (Bove & Garnier, 1998; Kirkpatrick, 1997). They are associated with several hundred plant diseases (McCoy et al., 1989). Phylogenetic analyses of phytoplasmal 16s rRNA and ribosomal protein genes have revealed that these organisms constitute a genus-level taxon (Gundersen et al., 1994, Seemüller et al., 1994). Approximately 15 groups (putative species) of phytoplasmas and several sub-groups have been delineated on the basis of differences in 16s rDNA sequences (Gundersen et al., 1994; Lee et al., 1993b; Namba et al., 1993; Schneider et al., 1995b; Seemüller et al., 1994). This delineation is supported by sequence differences in ribosomal protein genes (Gundersen et al., 1994; Lee et al., 1998) and the gene encoding elongation factor Tu (Schneider et al., 1997). Several of the groups were previously known from DNA-DNA hybridization data (Lee et al., 1990, 1991, 1992, 1993a; Nakashima et al., 1993). An AshY phytoplasma group was identified on the basis of hybridization data (Davis et al., 1992; Griffiths et al., 1994) and later from analyses of 16s rDNA from strain AshYIT compared with samples representing other known groups (Gundersen et al., 1994; Schneider et al., 1995b; Seemüller et al., 1994). Phytoplasmas associated with AshY and LWB are in the same group (Griffiths et al., 1994; Hibben et al., 1991) and are grafted-transmissible between Fraxinus and Syringa (Hibben et al., 1991).

Variability in rDNA sequences occurs within those phytoplasma groups in which multiple strains have been studied; sub-groups have been designated to categorize the variants. Some of the variability is associated with differing plant hosts or geographic origins of the strains. For example, phytoplasmas related to those associated with X-disease of Prunus (group 16SrIII of Lee et al., 1998) but differing from the reference strains CX and WX have been detected in various plants and assigned to eight sub-groups that are differentiated by RFLPs in 16s rRNA and ribosomal protein genes (Gundersen et al., 1996; Lee et al., 1998). These assignments imply that minor differences in sequence homology in the highly conserved 16s rRNA genes are associated with biologically important differences in other parts of the genome.

Strain AshYIT, alone, has represented the AshY phytoplasma group in classification research here-tofore (Davis et al., 1992; Lee et al., 1993b; Seemüller et al., 1994). This group is designated as 16SrVII in the scheme of Lee et al. (1998). Its closest known relatives, on the basis of phylogenetic analyses of rDNA, are phytoplasmas associated with brinjal little-leaf and clover proliferation (Gundersen et al., 1994; Schneider et al., 1995a; Seemüller et al. 1998). Other strains in the AshY group have not been studied in detail, and variability among them (Mäurer et al., 1993) is little known. The present research was undertaken with the goal of learning if phytoplasmas associated with AshY and LWB are a discrete, coherent taxon. Specific objectives were as follows: (1) to detect and characterize sequence variability in 16s rRNA genes of AshY and LWB phytoplasmas, (2) to determine whether variability in 16s rDNA is associated with other genomic differences and/or related to host or geographic origin, and (3) to ascertain whether reference strain AshYIT is typical of the group. These objectives were approached through analyses of phytoplasmal DNA collected from six plant species across the known range of AshY. Evidence is presented which indicates that the phytoplasmas associated with AshY and LWB constitute a coherent (though non-uniform) taxon, for which the name ‘Candidatus Phytoplasma fraxinii’ is proposed under guidelines implemented by the International Committee on Systematic Bacteriology for provisional classification of incompletely described micro-organisms (Murray & Stackebrandt, 1995).

METHODS

Phytoplasma collection and propagation. Nineteen samples of phytoplasma-infected Fraxinus or Syringa representing three species of each genus were obtained from naturally infected source plants in 16 localities between 71° and 113° W longitude (Massachusetts to Saskatchewan and Utah, Table 1). Most samples consisted of twigs with foliage; one sample was a root segment and one was a potted lilac. Phytoplasmal infection was initially detected or verified by means of the DAPI (4', 6-diamidino-2-phenylindole .2HCl) fluorescence test (Seemüller, 1976; Sinclair et al., 1996). Six samples collected from diseased ash were utilized directly for DNA extraction. Phytoplasmas from the other 13 source plants were transmitted by grafting to potted Fraxinus velutina Torr. (velvet ash) grown from seed and were maintained in this species. Twelve phytoplasma lines were further transmitted by Cuscuta subinclusa Dur. & Hilg. (dodder) to Catharanthus roseus (L.) G. Don. (periwinkle) grown from seed and then were maintained in this species by serial grafting. Phytoplasma populations maintained in ash or periwinkle are referred to hereafter as strains. All plants were grown in a screened greenhouse with periodic insecticide treatments to prevent unwanted introduction or transmission of phytoplasmas.

DNA extraction. The nucleic acid extraction procedure of Lee et al. (1993a) was used for ash samples, and the method of Dellaporta et al. (1983) was used for periwinkle samples, beginning with the grinding of 75 g fresh leaf midribs in liquid nitrogen. The final pellets were treated with RNase, and DNA concentrations were estimated spectrophotometrically using standard techniques (Sambrook et al., 1989).

DNA amplification. Phytoplasmal rDNA was amplified in either single or nested PCR reactions and then analysed with restriction endonucleases. Four primer pairs were used to obtain products as follows: R16F2/R2 (Lee et al., 1993b) for 1.2 kb of 16s rDNA; P1/P7 (Deng & Hiruki, 1991; Schneider et al., 1995b) for 1.8 kb comprising nearly the entire 16s rRNA gene and all of the 16s-23s spacer;
Table 1. Designations, origins and collectors of AshY and LWB phytoplasma strains and samples

<table>
<thead>
<tr>
<th>Strain designation</th>
<th>Origin</th>
<th>Host</th>
<th>Collector</th>
</tr>
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<tr>
<td>AshY1&lt;sup&gt;T&lt;/sup&gt;</td>
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<td><em>Fraxinus americana</em></td>
<td>J. A. Matteoni</td>
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<tr>
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<td>W. A. Sinclair</td>
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<td>W. A. Sinclair</td>
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<td>C. R. Hibben</td>
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<td><em>Fraxinus pennsylvanica</em></td>
<td>C. L. Ash</td>
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<td>C. L. Ash</td>
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<td>W. A. Sinclair</td>
</tr>
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<td><em>Fraxinus pennsylvanica</em></td>
<td>W. A. Sinclair</td>
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<td>W. A. Sinclair</td>
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<td><em>Syringa × prestoniae</em></td>
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<td>LWB2</td>
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<td><em>Syringa × josiflexa</em></td>
<td>C. R. Hibben</td>
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<td>LWB3</td>
<td>Boston, MA, USA</td>
<td><em>Syringa patula</em></td>
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<td><em>Fraxinus pennsylvanica</em></td>
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<td>None</td>
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<td>Fargo, ND, USA</td>
<td><em>Fraxinus pennsylvanica</em></td>
<td>J. A. Walla</td>
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<td>None</td>
<td>Palisade, NJ, USA</td>
<td><em>Fraxinus americana</em></td>
<td>R. Jomantiene</td>
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<td>None</td>
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<td><em>Fraxinus pennsylvanica</em></td>
<td>D. Reynard</td>
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<tr>
<td>None</td>
<td>Saskatoon, SK, Canada</td>
<td><em>Fraxinus pennsylvanica</em></td>
<td>D. Reynard</td>
</tr>
</tbody>
</table>

RFLP analyses. Aliquots of the PCR product obtained with primer pair R16F2/R2 from each of 12 strains in periwinkle and a sample from *Fraxinus americana* (New Jersey) were digested at 37 °C overnight with restriction enzyme *AluI* to determine whether all had the same RFLP profile as reference strain AshY1<sup>T</sup>. This *AluI* profile is unique to group 16SrVII of Lee et al. (1993b). Variability among AshY or LWB phytoplasmas in 16S rDNA and in the 16S–23S spacer was studied by digesting aliquots of the PCR product obtained with primer pair P1/P7 from 11 strains, AshY1<sup>T</sup>–AshY8 and LWB1–LWB3, overnight at 37 °C with each of *AluI, DraI, EcoRI, EcoRII, HaeIII, HhaI, HinfI, HpaI, HpaII, KpnI, MboI, MseI, PstI, RsaI* and ThaI (Gibco-BRL) or at 65 °C overnight with *TagI*. Each digest used 10 U enzyme in the presence of spermidine (40 mM) in a 10 μl reaction. The restriction products were separated by electrophoresis in a 6-5 or 9% polyacrylamide gel prepared in TBE (Sambrook et al., 1989) and stained with ethidium bromide. DNA bands were visualized as described above.

P1/P7 or B1/rAshYs amplifiers from two additional AshY phytoplasma strains and six other AshY phytoplasma collections were examined for conformity to RFLP profile types that had been identified by the above procedure. Samples were analysed with *AluI, HhaI* and *TagI* as described above. Amplifiers from strains AshY1<sup>T</sup>, AshY3, AshY5 and LWB3, which represented the four known RFLP profile types, were used as standards.

Variability in the ribosomal protein operon of strains AshY1<sup>T</sup>, AshY3, AshY5 and LWB3 was assessed by digesting PCR products obtained using primer pair rpFl/R1 at 37 °C overnight with *AluI, DraI, HhaI, MseI and RsaI*, or at 65 °C overnight with *TagI*. After RFLPs were detected with only *MseI*, the rpFl/R1 products from seven additional strains were digested separately with this enzyme and with *AluI*. 

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*B1/rAshYs (Smart et al., 1996) for 1.5 kb comprising portions of the 16S rRNA gene and 16S–23S spacer; and rpFl/R1 (Lim & Sears, 1992) for 1.24 kb of the ribosomal protein gene operon. Primary (direct) PCRs were utilized throughout, except that five DNA samples from green ash in the Great Plains and Rocky Mountain region were subjected to nested PCRs using primer pair P1/P7 in the primary reaction and B1/rAshYs in the nested reaction. This was done because the primary PCR products, although discernible by electrophoresis, were insufficient for RFLP analysis. PCR's with primer pairs R16F2/R2, P1/P7 and rpFl/R1 were performed as previously described (Lee et al., 1993b, 1998) in 50 μl mixtures containing 100 ng nucleic acid and 1.25 U Taq DNA polymerase (Gibco-BRL) with the following concentrations of other reagents: each deoxynucleoside triphosphate, 200 μM; each primer, 0.4 μM; 1 × Buffer II (Perkin-Elmer); and MgCl<sub>2</sub>, 3.5 mM. PCR amplifications were performed using an automated thermocycler (Perkin Elmer DNA Thermal Cycler (480)). Parameters used with primer pairs R16F2/R2 and rpFl/R1 for 35-cycle PCRs were denaturation at 94 °C for 1 min (2 min for the first cycle), annealing for 2 min at 50 °C, and primer extension for 3 min (10 min for last cycle) at 72 °C. Parameters used for 35-cycle PCRs with primer pair P1/P7 were denaturation at 94 °C for 30 s (90 s for the first cycle), annealing for 55 s at 55 °C, and primer extension for 80 s (9 min for the last cycle) at 72 °C. PCRs with primer pair B1/rAshYs were performed as described by Smart et al. (1996), except that 100 ng template was used per 30 μl reaction and denaturation in the first cycle was at 94 °C for 5 min. PCR products (5 μl) were detected and their sizes estimated by electrophoresis with size standards in 0.7% agarose gel in TBE buffer, followed by staining with ethidium bromide and visualization of the DNA bands with a UV transilluminator.
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Fig. 1. RFLP analyses of phytoplasmal 16S rDNA, amplified by PCR with primer pair R16F2/R2, from ash and lilac affected by AshY and LWB, respectively. PCR products were digested with Alul. Lanes S1 and S2 contained 1 kb and 100 bp ladders, respectively (Gibco-BRL).

Nucleotide sequencing, sequence alignment and cladogram construction. The P1/P7 amplimers from strains AshY1, AshY3, AshY5, AshY7 and LWB3 were sequenced, because these strains represented the four RFLP profiles encountered in 16S rDNA among AshY and LWB strains. Strains AshY5 and AshY7 had the same profile. The PCR products were purified using 0.7% low-melting-point agarose (Ultrapure LMP; Gibco-BRL) prepared in TAE (40 mM Tris acetate, 2 mM EDTA) and extracted with the QiAquick gel extraction kit (Qiagen). The final elution from the column was performed with sterile distilled water. DNA concentrations were estimated spectrophotometrically and adjusted for sequencing using sterile distilled water. Sequencing was performed using automated equipment in Cornell University’s Biotechnology Center. Each strand was sequenced with at least five primers located along the template to allow overlapping of sequences and thus eliminate ambiguous regions. Sequence data were collated, aligned and mapped using LASERGENE software (DNASTAR, 1997). The sequences were aligned with the aster yellows sequence of Lim & Sears (1989). The locations of restriction enzyme recognition sites reported here are based on that sequence.

Cladistic analysis and phylogenetic tree construction were performed with PAUP version 3.1.1 (Swofford, 1993) and MACCLADE (Maddison & Maddison, 1992). Data from the four distinct AshY strain sequences plus 12 other phytoplasma and two Acholeplasma 16S rDNA sequences obtained from GenBank were used. Acholeplasma laidlawii was selected as the outgroup to root the tree. The tree was constructed using a heuristic search with random stepwise addition, implementing the tree bisection and reconnection branch-swapping algorithm to find the optimum arrangement. The analysis was replicated 100 times.

Southern hybridizations. Total DNA from periwinkle plants, either healthy or infected with phytoplasma strain AshY1, AshY3, AshY5, AshY8 or LWB3, were analysed. Samples (4 µg) were doubly digested with restriction endonucleases EcoRI and HindIII (Gibco-BRL) at 37 °C overnight. Digested samples were electrophoresed in 0.7% agarose gel and transferred to Hybond-N+ membranes (Amersham) under alkaline conditions (Sambrook et al., 1989). Single randomly cloned genomic DNA fragments from each of phytoplasma strains BB (tomato big bud; Lee et al., 1990), CP (clover proliferation; Lee et al., 1991) and EY1 (elm yellows; Lee et al., 1993a), designated BB111, CP67 and EY24, respectively, were used as probes. The cloned fragments were approximately 2.2 kb, 3.0 kb and 1.2 kb in size, respectively. Probe BB111 was labelled with biotin, using the BioNick labelling system (Gibco-BRL), and detected using

Fig. 2. RFLP analyses of phytoplasmal rDNA, amplified by PCR with primer pair P1/P7, from ash and lilac affected by AshY and LWB, respectively. PCR products were digested with (a) Alul, (b) Hhal or (c) TaqI. Lane S3 contained PCR Marker (Sigma).
RESULTS

DNA amplification and RFLP analyses

The AluI profiles obtained from R16F2/R2 products of all 12 AshY and LWB strains tested and from the sample from Fraxinus americana in New Jersey were identical to the published profile of AshYIT, which is unique to the AshY phytoplasma group (Lee et al., 1998) (Fig. 1). The AluI recognition sites occur at positions 234, 291, 1002 and 1249 in the AshYIT 16S rRNA gene sequence.

Variations in RFLP profiles of P1/P7 digests of the AshY strains and collections were observed with AluI, HhaI and TaqI (Fig. 2) but not with the other enzymes. Four RFLP profile types were differentiated: profile A, which was shown by AshYIT and five additional strains and collections; profile B, shown by AshY5 and 10 additional strains and collections; profile C, unique to LWB3; and profile D, unique to AshY3 (Table 2). The locations of restriction sites for AluI, HhaI and TaqI were mapped using sequence data from strains AshYIT, AshY3, AshY5 and LWB3 (Fig. 3). With AluI, the strains separated into two groups with profiles similar to those of AshYIT or AshY5 (Fig. 2a). The latter strain lacks an AluI site that AshYIT possesses. With HhaI, AshY3 and LWB3 had unique profiles, while all other strains had the profile of AshYIT (Fig. 2b). AshY3 had an additional restriction site for HhaI just upstream from the tRNA" motif in the 16S–23S spacer (Fig. 3). LWB3 had one less restriction site for HhaI than did AshYIT. With TaqI, all strains except AshY3 had the same profile (Fig. 2c). AshY3 lacked a TaqI site that the other strains possessed.

The nested PCR with DNA from green ash in the Great Plains and Rocky Mountain region (Durango, CO; Bridger, MT; Fargo, ND; and Regina and Saskatoon, SK, Canada) yielded the expected 1·5 kb products (Smart et al., 1996). The amplimers were

<table>
<thead>
<tr>
<th>Strain and origin</th>
<th>Profile with restriction enzyme:</th>
<th>Profile type</th>
<th>GenBank no.</th>
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<td>A</td>
<td>AF092209</td>
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<td>AshY4, NY, USA</td>
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<td>LWB1, NY, USA</td>
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</tr>
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</table>
digested with AluI, HhaI and TaqI. The profiles from all samples were identical to that of strain AshY5 (data not shown).

Restriction enzyme digests of PCR products from the ribosomal protein DNA fragment were identical for all strains with all enzymes used except MseI. The profiles obtained with this enzyme allowed strain AshY3 to be differentiated from the other strains. AshY3 DNA lacked a band at about 180 bp that profiles of the other strains possessed; it also had an extra band at less than 75 bp (Fig. 4).

Nucleotide sequences

Alignment of a 1423 bp sequence of the 16S rRNA gene of strains AshYIT, AshY3, AshY5, AshY7 and LWB3 revealed four genotypes corresponding to the RFLP profile groups detected with AluI, HhaI and TaqI. The sequence of AshY7 was identical to that of AshYS. The sequences varied at only three positions, where single base substitutions defined or abolished recognition sites for the three enzymes. These sites were a TaqI site (TCGA) at position 212, an HhaI site (GCGC) at position 239 and an AluI site (AGCT) at position 1435 (Fig. 3). Positions 212 and 239 are within a variable region of the gene; position 1435 is in a conserved region (Lim & Sears, 1989). Type strain, AshY1T, had all three sites.

Phylogenetic relationships

A single most-parsimonious tree was obtained by analysis of partial 16S rDNA sequences of 16 phytoplasmas with A. laidlawii as an outgroup. The four AshY phytoplasma strains clustered tightly together (with fewer than four character changes among them) and this group differed from all others by a minimum of 20 character changes (Fig. 5). Phytoplasmas associated with brinjal little-leaf and clover proliferation were the most closely related to the AshY group; these phytoplasmas were followed by strain ULW (an elm-yellows phytoplasma).

Phytoplasma signature sequences and unique 16S rRNA sequences

The 16S rRNA of AshY phytoplasmas contains all six sequences previously reported as unique to phytoplasmas (Gundersen et al., 1994). In the AshY and LWB phytoplasmas studied, there were at least two sequences unique to this group. The 21 base sequence 5'-CGGAAACCCCCCCCTAAAGGTTC-3' beginning at position 66 differs at 3–13 positions from corresponding sequences of phytoplasmas in other groups. In comparisons of AshY phytoplasmas with their three closest relatives, there are base differences at positions 69, 75 and 77 for AshY1T versus BLL; at positions 69, 74 and 77 for AshY1T versus CP; and at positions 70, 75 and 85 for AshY1T versus ULW. The
No. of characters that change unambiguously on branch

0-4

5-9

10-14

15-19

20-24

25-29

30-34

35-39

40-44

> 45

Not calc

Ashy1
LWB3
AshY5
AshY3
BLL
CP
ULW
LWB
PPWB
CX
CYE
PnWB
SPWB
PD
AY1
BB
A. palmae
A. laidlawii

Fig. 5. Phylogenetic tree constructed by parsimony analysis of 16S rDNA sequences of four phytoplasma strains associated with Ashy or LWB and 12 reference organisms. The AshY and LWB strains represent the four RFLP profile types detected in this phytoplasma group (AshY1, AshY3, AshY5 and LWB3). Reference strain identities and EMBL or GenBank accession numbers are as follows: A. laidlawii, Acholeplasma laidlawii (M23932); A. palmae, Acholeplasma palmae (L33734); AY1, Maryland aster yellows (L33767); BB, tomato big bud (L33760); CP, clover proliferation (L33761); CX, Canadian peach X-disease (L33733); CYE, clover yellow edge (L33766); BLL, brinjal little-leaf (X83431); ULW, elm yellows (X68376); LWB, loofah witches'-broom (L33763); PD, pear decline (X76425); PnWB, peanut witches'-broom (L33765); PPWB, pigeon pea witches'-broom (L33735); SPWB, sweet potato witches'-broom (L33770).

sequence 5'-AGGAAAGTC-3' at positions 588–596 differs at two to five positions from corresponding sequences of phytoplasmas in other groups. Differences at positions 590 and 591 separate Ashy phytoplasmas from strains BLL, CP and ULW.

Southern hybridizations

Three of the five strains examined were differentiated by analysis with EcoRI and HindIII. Two RFLP profiles were obtained with probe CP67 and three with BB111 (Fig. 6a, b). Probe CP67 hybridized with two DNA bands from strain AshY1, and one band of consistent size from strains AshY5, AshY8 and LWB3. This probe did not hybridize with DNA of AshY3. Probe BB111 hybridized with DNA from all five strains. AshY1 and AshY3 had unique profiles, while AshY5, AshY8 and LWB3 had profiles that were identical to each other. The RFLP profiles of all AshY strains tested on membranes probed with EY24 were identical (data not shown).

Genome size

Strain AshY3 had an estimated genome size of 645 kbp. The DNA preparation from AshY1 (which had been extracted from declining periwinkle plants) proved unsatisfactory for analysis.

DISCUSSION

Evidence from RFLP and sequence analyses of 16S rRNA genes, RFLP analysis of amplified portions of
ribosomal protein genes, and Southern analysis of chromosome DNA supports the view that AshY and LWB phytoplasmas constitute a coherent but heterogeneous taxon, probably at species level. Phylogenetic analysis of 16S rDNA sequences produced a tree that was in agreement with those of Gundersen et al. (1994), Schneider et al. (1995a, b) and Seemüller et al. (1998), except that the AshY group is now represented by three AshY strains and one LWB strain. Phytoplasma strain AshY1T may be considered the type of its taxon, as its DNA possesses all three of the restriction-enzyme recognition sites that define, by their presence or absence, the known variability in 16S rDNA of the AshY group. The LWB strains, of which we studied three (LWB1, LWB2 and LWB3), are closely related to AshY1T. LWB1 and LWB2 had rDNA RFLP profiles identical to that observed for AshY1T. The rDNA of LWB3 had a unique RFLP profile. This phytoplasma was from a lilac in an arboretum in Boston, MA. The history of the plant, and thus the origin of the phytoplasma, was unknown. There is, to date, only one report of a phytoplasma, in a plant other than<br />Fraxinus or Syringa, that may belong to the AshY taxon; a possible member was detected in Prunus avium L. (Rosaceae) in China (Li et al., 1997).

Four 16S rDNA RFLP profile types were found within the AshY group, and the corresponding restriction sites were located by sequence analysis. One site was in the conserved region of the gene and two were in a variable region. Each of 13 AshY phytoplasma strains and six additional collections conformed to one of these profile types. Sequence homology in 16S rDNA plus the 16S–23S spacer among AshY and LWB strains representing the four RFLP profile types was >99.8 %. In contrast, 16S rDNA sequence homology between strain AshY1T and the most closely related phytoplasma in a different group (brinjal little-leaf, BLL) was 96.5 %, in close agreement with the 97.2 % reported by Schneider et al. (1995a), who used a different computer program for sequence comparisons. Strains with the rDNA RFLP profile of AshY1T were, with one exception, all collected in New York and Ontario and occurred in both ash and lilac. Strains similar to AshY5 were, with two exceptions, collected in the Great Lakes region and westward and were all from ash. Three localities, Ithaca–Enfield (NY), Ann Arbor (MI) and St Paul (MN), were represented by two to three strains each; strains from within localities were indistinguishable. Strains AshY3 from F. velutina in Utah and LWB3 from Syringa patula in Massachusetts were the only representatives from those regions and were unique.

The finding of only two RFLP profile variants in the ribosomal protein gene operon was unexpected because the strains examined represented the four 16S rDNA restriction profile types and because Gundersen et al. (1996) had found greater variability in the ribosomal protein gene operon than in the 16S rRNA genes of phytoplasma groups 16SrI and 16SrIII (aster yellows and Prunus X-disease groups, respectively). Sequence analysis of the ribosomal protein gene operon of AshY phytoplasma strains might reveal additional variation. However, the similarity detected by RFLP analysis is consistent with the interpretation of AshY phytoplasmas as a coherent, species-level taxon.

The finding of genetic variation with every assay in this work raises the question of whether the AshY phytoplasma group includes discrete sub-groups and, if so, whether they are represented by the four 16S rDNA genotypes. Representatives of three of these genotypes were differentiated by Southern analyses of total phytoplasma DNA, hybridized with either probe pBB111 or probe pCP67. AshY1T and AshY3 were distinct from each other and from a group comprising AshY5 and AshY8 (both of which have the same 16S rDNA genotype) and LWB3. As both probes were known to hybridize to the DNA of various phytoplasmas (Lee et al., 1990), they may represent conserved sequences. Therefore, the differentiation of strains by analysis of both rDNA and unidentified chromosomal DNA may indicate the existence of genetically differentiated sub-groups. Mäurer et al. (1993) noticed three RFLP profiles in ash phytoplasma DNA probed with anonymous fragments from an elm-yellows phytoplasma. Samples from Michigan had one profile, while those from a region extending from eastern Ohio into central New York had a second profile, and strain AshY1T a third. Our findings, coupled with those of Mäurer et al. (1993), indicate a possible correlation between RFLP profile type and geographic source. More extensive sampling, with attention to host and geographic origins, would be necessary to determine if genetically distinct populations of AshY phytoplasmas predominate in particular regions. Even if differentiated populations occur, however, some mixing would be anticipated as a result of phytoplasma transport by alate vectors and by host plants during commerce. At present, given the close relationships of all the strains studied and those of their hosts, it seems inappropriate to designate AshY phytoplasma sub-groups formally.

The apparent size of the AshY3 chromosome, 645 kbp, is among the smaller sizes reported for phytoplasmas or other mollicutes (Neimark & Kirkpatrick, 1993; Neimark & Carle, 1995) and evidently is not a characteristic by which AshY phytoplasmas can be distinguished from others. Three strains of the Prunus X-disease phytoplasma group had estimated chromosome sizes within the range 640–650 kbp (Neimark & Kirkpatrick, 1993). Moreover, given the variation detected in other characteristics of AshY phytoplasmas, we cannot assume that the same chromosome size would be found for AshY1T or other strains.

The AshY phytoplasma group is apparently a monophyletic lineage distinct from other phytoplasmas at the putative level of species. This interpretation is consistent with previous analyses involving only strain, AshY1T, and with the putative genus-level status of
phytoplasmas in comparisons with cultivable mollicutes (Gundersen et al., 1994). It is desirable to have a name by which the group can be known. As the International Committee on Systematic Bacteriology has implemented a scheme for assigning incompletely described prokaryotes to the provisional status Candidatus (Murray & Stackebrandt, 1995), the name 'Candidatus Phytoplasmata fraxinii' is hereby proposed for phytoplasmas in the AshY group, including those associated with LWB. This taxon has the following description: [(Mollicutes) NC; NA; O, wall]-less; NAS (GenBank accession no. AF092209); oligonucleotide sequences of unique regions of the 16S rRNA gene S'-CGGAAAAACCCCTAAAAGGT-3' and 5'-AGGAAAAAGTC-3'; P (phloem of Fraxinus, Syringa): M]. Type strain AshY1T and other strains mentioned herein, maintained in Catharanthus roseus, are available from the authors.

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