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

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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 *Alu*I, *Hha*I or *Taq*I, for a total of four RFLP profile types. Sequencing of the amplimers from strains AshY1^T, 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/R1 from each of the four strains noted above, was analysed with six restriction enzymes, resulting in the detection of two RFLP profiles with *Mse*I. 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^T, 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^T. 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; PD, pear decline; 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 rRNA gene and the 16S–23S spacer) of phytoplasma strains AshY1^T, AshY3, AshY5 and LWB3 are AF092209, AF105315 and AF105316, 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 (Bové & 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 AshY1^T 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 graft-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 AshY1^T, alone, has represented the AshY phytoplasma group in classification research heretofore (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 AshY1^T 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 fraxini' 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 directSly 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 hereinafter 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 0·75 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

Strain designation	Origin	Host	Collector
AshY1^T	Ithaca, NY, USA	*Fraxinus americana*	J. A. Matteoni
AshY2	Enfield, NY, USA	*Fraxinus americana*	W. A. Sinclair
AshY3	Springdale, UT, USA	*Fraxinus velutina*	W. A. Sinclair
AshY4	Verbank, NY, USA	*Fraxinus americana*	C. R. Hibben
AshY5	St Paul, MN, USA	*Fraxinus pennsylvanica*	C. L. Ash
AshY6	St Paul, MN, USA	*Fraxinus pennsylvanica*	C. L. Ash
AshY7	Ann Arbor, MI, USA	*Fraxinus americana*	W. A. Sinclair
AshY8	Ann Arbor, MI, USA	*Fraxinus pennsylvanica*	W. A. Sinclair
AshY11	Ames, IA, USA	*Fraxinus pennsylvanica*	W. A. Sinclair
AshY12	Inwood, WV, USA	*Fraxinus americana*	W. A. Sinclair
LWB1	Ithaca, NY, USA	*Syringa* × *prestoniae*	W. A. Sinclair
LWB2	Ottawa, ON, Canada	*Syringa* × *josiflexa*	C. R. Hibben
LWB3	Boston, MA, USA	*Syringa patula*	J. H. Alexander, III
None	Bridger, MT, USA	*Fraxinus pennsylvanica*	J. Scianna
None	Durango, CO, USA	*Fraxinus pennsylvanica*	W. R. Jacobi
None	Fargo, ND, USA	*Fraxinus pennsylvanica*	J. A. Walla
None	Palisade, NJ, USA	*Fraxinus americana*	R. Jomantiene
None	Regina, SK, Canada	*Fraxinus pennsylvanica*	D. Reynard
None	Saskatoon, SK, Canada	*Fraxinus pennsylvanica*	D. Reynard

fB1/rAshYs (Smart *et al.*, 1996) for 1·5 kb comprising portions of the 16S rRNA gene and 16S–23S spacer; and rpF1/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 fB1/rAshYs in the nested reaction. This was done because the primary PCR products, although discernible by electrophoresis, were insufficient for RFLP analysis.

PCRs with primer pairs R16F2/R2, P1/P7 and rpF1/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 $MgCl_2$, 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 rpF1/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 fB1/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.

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 *Alu*I to determine whether all had the same RFLP profile as reference strain AshY1^T. This *Alu*I 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^T–AshY8 and LWB1–LWB3, overnight at 37 °C with each of *Alu*I, *Dra*I, *Eco*RI, *Eco*RII, *Hae*III, *Hha*I, *Hin*fI, *Hpa*I, *Hpa*II, *Kpn*I, *Mbo*I, *Mse*I, *Pst*I, *Rsa*I and *Tha*I (Gibco-BRL) or at 65 °C overnight with *Taq*I. 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 fB1/rAshYs amplimers 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 *Alu*I, *Hha*I and *Taq*I as described above. Amplimers from strains AshY1^T, 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^T, AshY3, AshY5 and LWB3 was assessed by digesting PCR products obtained using primer pair rpF1/R1 at 37 °C overnight with *Alu*I, *Dra*I, *Hha*I, *Mse*I and *Rsa*I, or at 65 °C overnight with *Taq*I. After RFLPs were detected with only *Mse*I, the rpF1/R1 products from seven additional strains were digested separately with this enzyme and with *Alu*I.

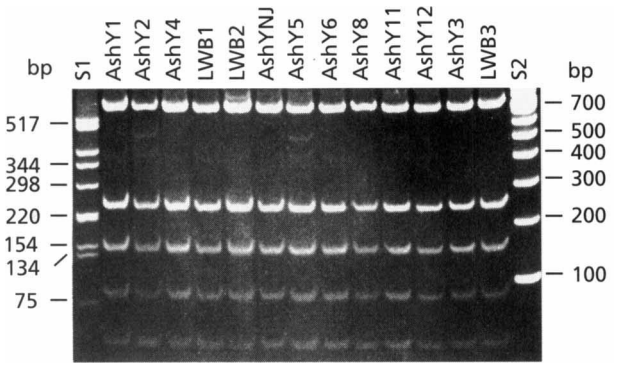

Fig. 1. RFLP analyses of phytoplasmal 16S rDNA, amplified by PCR with primer pair R16F2/R2, from ash and lilac affected by AshY and LWB, respectively. PCR products were digested with *Alu*I. Lanes S1 and S2 contained 1 kb and 100 bp ladders, respectively (Gibco-BRL).

Nucleotide sequencing, sequence alignment and cladogram construction. The P1/P7 amplimers from strains AshY1^T, AshY3, AshY5, AshY7 and LWB3 were sequenced, because these strais 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^T, AshY3, AshY5, AshY8 or LWB3, were analysed. Samples (4 μg) were doubly digested with restriction endonucleases *Eco*RI and *Hin*dIII (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),

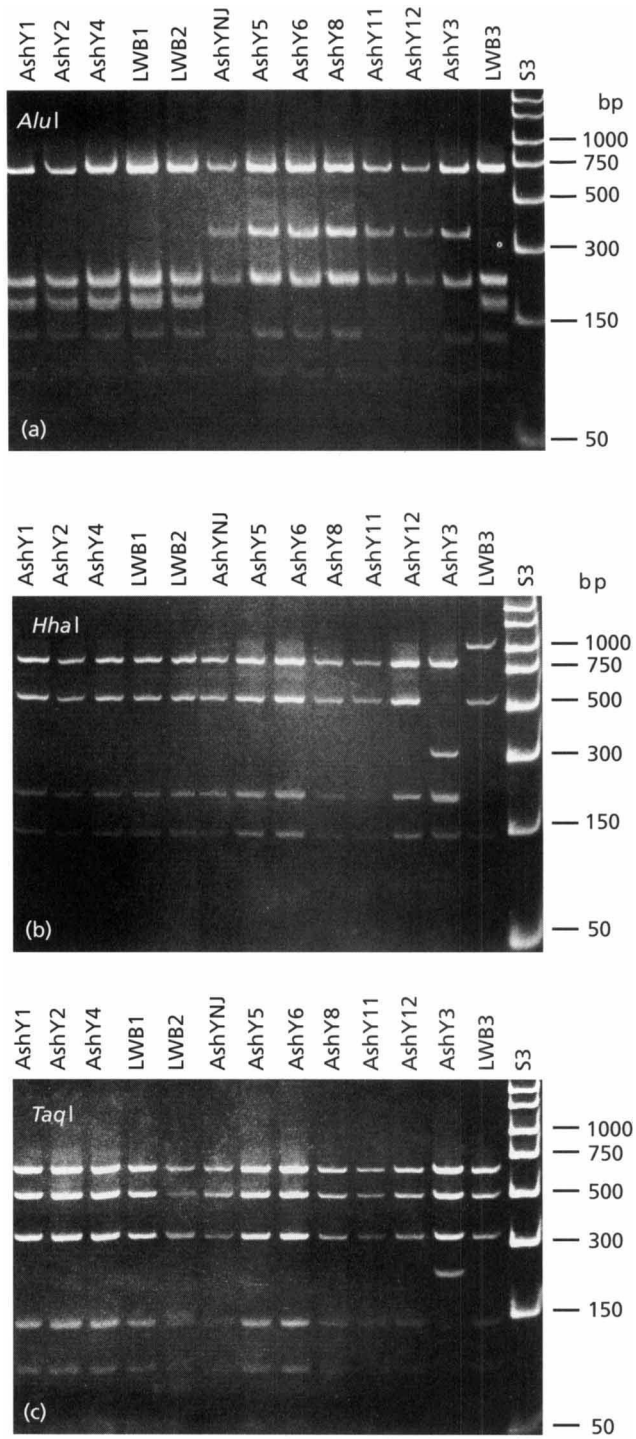

Fig. 2. RFLP analyses of phytoplasmal rDNA, amplified by PCR with primer pair P1/P7, from ash and lilac affected by AshY and LWB, respectively. PCR products were digested with (a) *Alu*I, (b) *Hha*I or (c) *Taq*I. Lane S3 contained PCR Marker (Sigma).

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

the BluGENE non-radioactive nucleic acid detection system (Gibco-BRL). Manufacturers' protocols were followed for hybridization and detection. Probes CP67 and EY24 were labelled with ^{32}P using the Random prime DNA labelling system (Gibco-BRL). Unincorporated counts were separated using Microbiospin columns (Bio-Rad). Hybridization solutions contained 5 × SSC (20 × SSC is 3 M NaCl, 0·3 M Na_3citrate.$2H_2O$, pH 7·0), 5 × Denhardt's solution, 50 mM sodium phosphate (pH 7), 10 mM EDTA and 0·5 % SDS (Sambrook *et al.*, 1989). Prehybridization and hybridization were performed at 65 °C. Excess label was removed with 2 × SSC and 0·1 % SDS for 15 min at 65 °C; this was followed by two additional washes in 0·5 × SSC and 0·1 % SDS, each for 15 min at 65 °C. Autoradiography films with intensifying screens were exposed to the membranes for 48 h at −80 °C.

Genome size estimation. DNA was extracted and phytoplasmal chromosomes isolated as described by Firrao *et al.* (1996) from midveins of periwinkle plants singly infected with strains AshY1^T and AshY3 and also from a healthy plant in order to overcome the problems of contaminating plant chromosomal DNA. The chromosomes were linearized using gamma irradiation (Neimark & Lange, 1990). Fragment sizes were estimated by comparing their mobility with that of standards in PFGE (Neimark & Carle, 1995; Neimark & Kirkpatrick, 1993).

RESULTS

DNA amplification and RFLP analyses

The *Alu*I 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 AshY1^T, which is unique to the AshY phytoplasma group (Lee *et al.*, 1998) (Fig. 1). The *Alu*I recognition sites occur at positions 234, 291, 1002 and 1249 in the AshY1^T 16S rRNA gene sequence.

Variations in RFLP profiles of P1/P7 digests of the AshY strains and collections were observed with *Alu*I, *Hha*I and *Taq*I (Fig. 2) but not with the other enzymes. Four RFLP profile types were differentiated: profile A, which was shown by AshY1^T 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 *Alu*I, *Hha*I and *Taq*I were mapped using sequence data from strains AshY1^T, AshY3, AshY5 and LWB3 (Fig. 3). With *Alu*I, the strains separated into two groups with profiles similar to those of AshY1^T or AshY5 (Fig. 2a). The latter strain lacks an *Alu*I site that AshY1^T possesses. With *Hha*I, AshY3 and LWB3 had unique profiles, while all other strains had the profile of AshY1^T (Fig. 2b). AshY3 had an additional restriction site for *Hha*I just upstream from the tRNA^{Ile} motif in the 16S–23S spacer (Fig. 3). LWB3 had one less restriction site for *Hha*I than did AshY1^T. With *Taq*I, all strains except AshY3 had the same profile (Fig. 2c). AshY3 lacked a *Taq*I 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 2. Summary of RFLP analyses of phytoplasmal rDNA, amplified by PCR with primer pair P1/P7, from ash or lilac affected by AshY or LWB, respectively

Strain and origin	Profile with restriction enzyme:			Profile type	GenBank no.
	*Alu*I	*Hha*I	*Taq*I		
AshY1^T, NY, USA	1	1	1	A	AF092209
AshY2, NY, USA	1	1	1	A	
AshY4, NY, USA	1	1	1	A	
LWB1, NY, USA	1	1	1	A	
LWB2, ON, Canada	1	1	1	A	
Fargo, ND, USA	1	1	1	A	
AshY5, MN, USA	2	1	1	B	AF105316
AshY6, MN, USA	2	1	1	B	
AshY7, MI, USA	2	1	1	B	
AshY8, MI, USA	2	1	1	B	
AshY11, IA, USA	2	1	1	B	
AshY12, NJ, USA	2	1	1	B	
Bridger, MT, USA	2	1	1	B	
Durango, CO, USA	2	1	1	B	
Palisade, NJ, USA	2	1	1	B	
Regina, SK, Canada	2	1	1	B	
Saskatoon, SK, Canada	2	1	1	B	
LWB3, MA, USA	1	3	1	C	AF105317
AshY3, UT, USA	2	2	2	D	AF105315

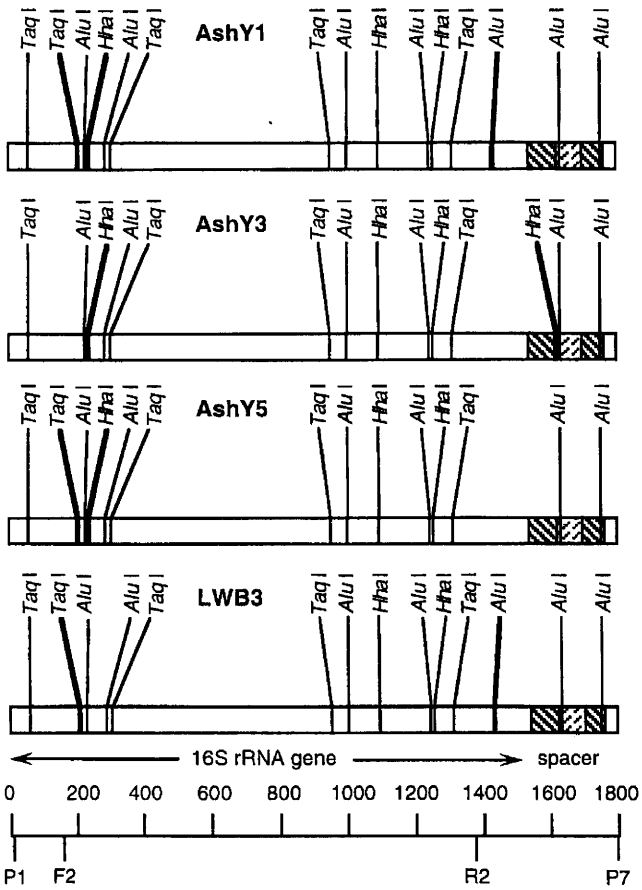

Fig. 3. Restriction maps of four 16S rDNA RFLP profile types obtained from AshY or LWB phytoplasma strains and distinguished by digestion with *Alu*I, *Hha*I and *Taq*I. Diagnostic restriction sites that distinguish the four types are indicated by bold lines.

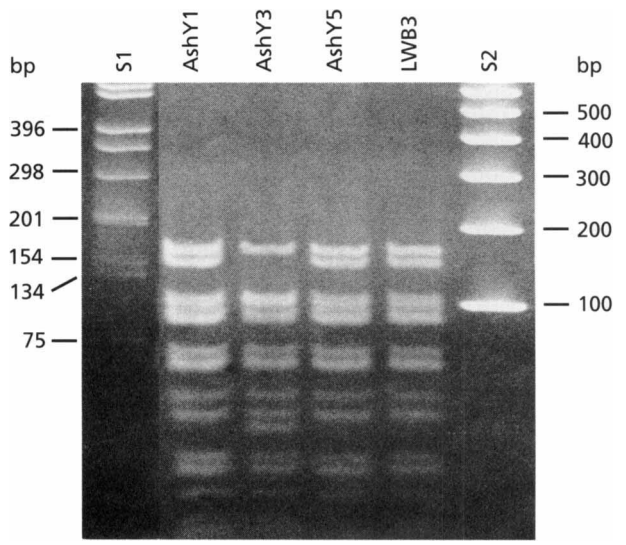

Fig. 4. RFLP analyses of phytoplasmal ribosomal protein DNA, amplified by PCR with primer pair rpF1/R1, from ash and lilac affected by AshY and LWB, respectively. PCR products were digested with *Mse*I. Lanes S1 and S2 contained 1 kb and 100 bp ladders, respectively (Gibco-BRL).

digested with *Alu*I, *Hha*I and *Taq*I. 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 *Mse*I. 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 AshY1^T, AshY3, AshY5, AshY7 and LWB3 revealed four genotypes corresponding to the RFLP profile groups detected with *Alu*I, *Hha*I and *Taq*I. The sequence of AshY7 was identical to that of AshY5. The sequences varied at only three positions, where single base substitutions defined or abolished recognition sites for the three enzymes. These sites were a *Taq*I site (TCGA) at position 212, an *Hha*I site (GCGC) at position 239 and an *Alu*I 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, AshY1^T, 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′-CGGAAACCCCTCAAAAGGTTT-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 AshY1^T versus BLL; at positions 69, 74 and 77 for AshY1^T versus CP; and at positions 70, 75 and 85 for AshY1^T versus ULW. The

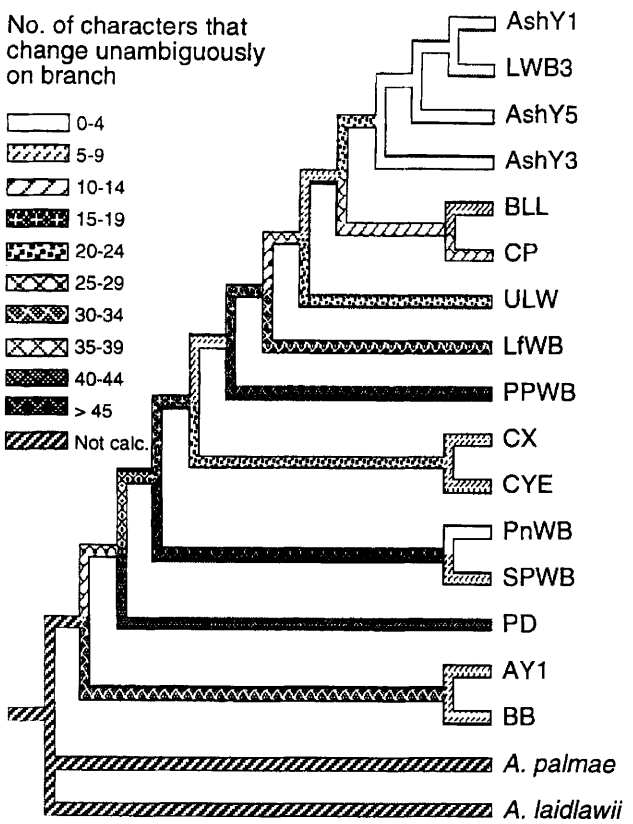

Fig. 5. Phylogenetic tree constructed by parsimony analysis of 16S rDNA sequences of four phytoplasma strains associated with AshY or LWB and 12 reference organisms. The AshY and LWB strains represent the four RFLP profile types detected in this phytoplasma group (AshY1^T, AshY3, AshY5 and LWB3). Reference 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); LfWB, 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 *Eco*RI and *Hin*dIII. 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^T 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^T 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).

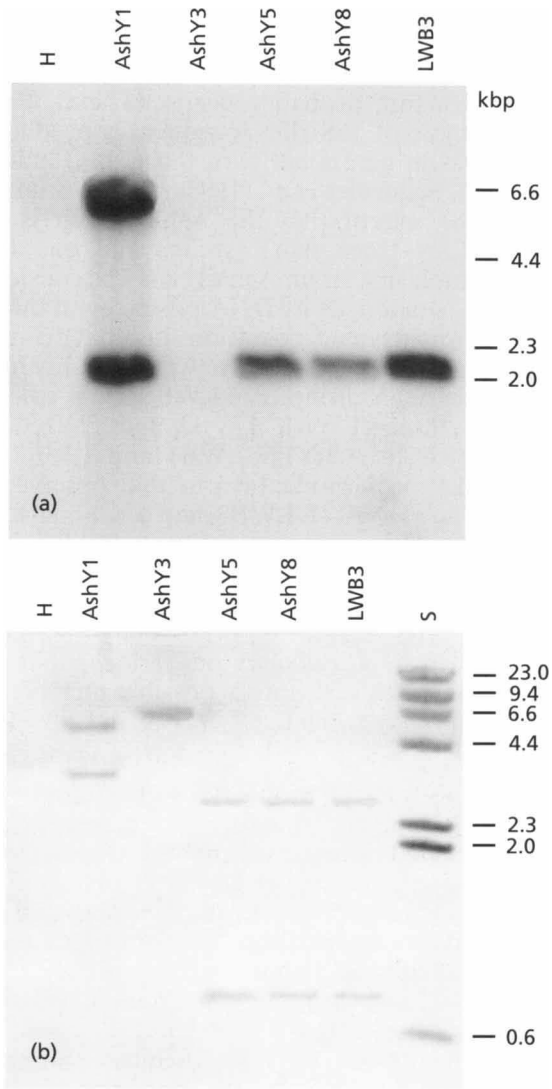

Fig. 6. Southern hybridization analysis of genomic DNAs prepared from *Catharanthus roseus* that was either healthy (H) or infected with phytoplasma strains associated with AshY or LWB. DNA samples were digested with *Eco*RI and *Hin*dIII and hybridized (a) with cloned ^{32}P-labelled probe CP67 from the clover proliferation (CP) phytoplasma or (b) cloned biotin-labelled probe BB111 from tomato big bud phytoplasma strain BB. Size standards were lambda-phage DNA *Hin*dIII digests (Gibco-BRL).

Genome size

Strain AshY3 had an estimated genome size of 645 kbp. The DNA preparation from AshY1^T (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 chromosomal 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 AshY1^T 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 AshY1^T. LWB1 and LWB2 had rDNA RFLP profiles identical to that observed for AshY1^T. 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 *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 AshY1^T 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 AshY1^T 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 phytoplasmal DNA, hybridized with either probe pBB111 or probe pCP67. AshY1^T 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 phytoplasmal 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 AshY1^T 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 AshY1^T 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, AshY1^T, and with the putative genus-level status of

phytoplasmas in comparisons with culturable 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* Phytoplasma fraxini' 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 5′-CGGAAACCCCTCAAAAGGTTT-3′ and 5′-AGGAAAGTC-3′; P (phloem of *Fraxinus*, *Syringa*); M]. Type strain AshY1^T and other strains mentioned herein, maintained in *Catharanthus roseus*, are available from the authors.

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