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Detection and molecular characterisation of an alfalfa phytoplasma in Argentina that represents a new subgroup in the 16S rDNA Ash Yellows group ('*Candidatus* Phytoplasma fraxini')

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Abstract

A phytoplasma infecting alfalfa crops was detected and characterised in Argentina, the Argentinean Alfalfa witches′-broom (ArAWB) phytoplasma. Typical witches′-broom symptoms were observed in diseased alfalfa plants from fields of the 'Cuyo' region in the Andean midwest. Pleomorphic bodies were observed by electron microscopy in sieve tubes of the diseased plants. The results obtained from the sequence homology, similarity coefficients derived from RFLP of the 16S rDNA and phylogenetic analysis led us to include this phytoplasma in the 16Sr VII (Ash Yellows) group. However, the ArAWB phytoplasma showed several differences when compared to other members of group 16Sr VII. The RFLP analysis of partial 16S rRNA gene of two ArAWB isolates, digested with 16 restriction enzymes, showed differences between the ArAWB and the reference strain (AshY1^T) in six enzyme patterns. Restriction patterns unique for the group and an exclusive *Hinf*I restriction site were found in the ArAWB phytoplasma rDNA. Moreover, the similarity coefficients (0.92–0.86) were lower than those obtained among other group members. The significant differences detected suggested that this phytoplasma belonged to a subgroup different from those described so far. We propose therefore, that the ArAWB phytoplasma should be included in a new VII-C subgroup, closely related to the EriWB phytoplasma (VII-B) described in Brazil.

Introduction

Argentina has been one of the main producers of high quality bovine meat for several decades. Among the cultivated forage crops, alfalfa (*Medicago sativa*) is one of the most widespread species. Nearly 10 years ago, an alarming outbreak of severe witches′-broom symptoms was observed in the 'Cuyo' region of Argentina, Andean midwest, in alfalfa fields destined for seed production (Hijano and Fernandez, 1995). Affected plants showed shoot proliferation, severely reduced leaf size, chlorosis, general stunting, and flower abortion. The symptoms were associated with phytoplasma infection according to the symptomatology described by Stuteville and Erwin (1990).

Phytoplasmas are plant pathogenic bacteria that inhabit plant phloem and insects, mainly leafhoppers. They are unculturable organisms, and thus cannot be characterised by the traditional techniques used for bacterial classification (Bové and Garnier, 1998). Instead, molecular methods have proved to be more accurate in comparison to the other techniques for the detection and description of phytoplasmas from different hosts. A

comprehensive scheme for phytoplasma classification and phylogenetic relationships definition has been established based on RFLP and sequence analysis of the highly conserved 16S rRNA and ribosomal protein genes (Lee et al., 1998; Seemüller et al., 1998). As a result of this classification, up to 20 groups and more than 41 subgroups have been determined. Subgroups have been defined by phytoplasma strains that have one or more 16S rDNA RFLP patterns different to other members of the same group, and confirmed by delineated subclades based on phylogenetic analysis from 16S rRNA gene sequences (Seemüller et al., 1998). New groups and subgroups arise constantly as new phytoplasmas are identified and characterised (Montano et al., 2000; Barros et al., 2002; Jomantiene et al., 2002). According to the ICSB (1997), the phytoplasmas are considered at the genus level and, at least each group, has been proposed to represent one putative phytoplasma species (IRPCM Phytoplasma/Spiroplasma Working Team-Phytoplasma Taxonomy Group, 2004). Besides, as proposed for the classification of uncultured bacteria, the provisional *Candidatus* status is to be applied (ICSB, 1995).

Based on their sequence homology, some 16Sr groups are more homogeneous than others. The apple proliferation (16Sr X), aster yellows (16Sr I) and elm yellows (16Sr V) groups, for instance, are conformed by phytoplasmas that show high similarity in their 16S rRNA gene sequence; nevertheless, they could be grouped into subgroups (Seemüller et al., 1998; Marcone et al., 2000). Other groups show variability even among members of the same subgroup, such is the case of the ash yellows (16Sr VII), in which multiple strains have been defined (Griffiths et al., 1999; Barros et al., 2002). Moreover, the host range and geographic distribution patterns can differ among groups. Many of the defined subgroups have a narrow host range or even a single host, and many of them are geographically isolated. On the other hand, one phytoplasma strain can cause disease in various plant hosts, in different regions (Lee et al., 1998). Diseases caused by ash yellows group phytoplasmas have been mainly detected in north America and are restricted to *Syringa* and *Fraxinus* species (Griffiths et al., 1999). Recently, a new Ash Yellows strain affecting *Erigeron* sp. has been reported in Brazil (Barros et al., 2002), showing a wider distribution and host range of the group. There are cases in which a particular symptomatology on the same plant host can be associated with two or more unrelated phytoplasmas in different geographical regions. The witches′-broom symptomatology has been reported in different areas of the world infecting alfalfa crops: the Alfalfa witches′-broom phytoplasma (AWB) in Canada (Khadhair and Hiruki, 1995), the Italian Alfalfa witches′-broom (IAWB) in Italy (Marcone et al., 1997), the Alfalfa witches′-broom (AlfWB) in Oman (Khan et al., 2002) and recently the Australian Lucerne Yellows (ALuY) reported by Pilkington et al. (2003) in Australia. In a preliminary report we described the presence of an ash yellows group phytoplasma associated with witches′-broom disease of alfalfa in Argentina (Meneguzzi et al., 2001). The present study reports extended characterisation and phylogenetic relationships of this phytoplasma, based on collective RFLP patterns and 16S rRNA gene sequence analysis.

Materials and methods

Phytoplasma sources

Alfalfa (*Medicago sativa*) samples showing typical witches′-broom symptoms were collected from fields destined for seed production in the 'Cuyo' region (San Juan province, Argentina). One representative plant from Pocito and San Martín Departments were selected for this study. The pathogen, named Argentinean alfalfa witches′-broom (ArAWB) phytoplasma, was maintained by grafting to healthy alfalfa plants grown in a greenhouse. Phytoplasma infection was tested by PCR and the group affiliation was confirmed by PCR-RFLP. DNA from the AshY1^T strain of '*Candidatus* Phytoplasma fraxini' (kindly supplied by Dr. E. Seemüller) was used as a reference strain.

DNA purification and PCR amplification

DNA was isolated from symptomatic and healthy alfalfa plants, with 2% CTAB (hexadecyltrimethyl-ammonium bromide) buffer, following the Doyle and Doyle (1990) procedure. The DNA quality and concentration was estimated by spectrophotometer and agarose gel electrophoresis using standard techniques (Sambrook et al., 1989).

rDNA of AshY1^T and ArAWB phytoplasma isolates from Pocito and San Martín were amplified by direct PCR. Two primer pairs were used to obtain products as follows: R16F2/R16R2 (Lee et al., 1993) for a 1.2 kb fragment of the 16S rDNA gene and P1/P7 (Deng and Hiruki, 1991; Schneider et al., 1995) for a 1.8 kb fragment that comprises nearly the full 16S rDNA gene, complete 16S–23S spacer region and the 5′ end of the 23S gene. The PCRs were performed in a thermal cycler (Biometra TRIO-Thermoblock). The 40 μl reaction mixture using R16F2/R16R2 primer pair was performed according to Lee et al. (1993), except for the annealing conditions that were changed to 1 min at 58 °C. For P1/P7 primers, 35 cycles were conducted under the following parameters: 30 s (3 min for the first cycle) denaturation step at 95 °C, annealing for 30 s at 60 °C, and primer extension 1 min 40 s at 72 °C. Nested PCR was performed with the specific fB1/rASHYS (Smart et al., 1996) primer pair using a 1/50 dilution of a first round amplification with primers P1/P7. In order to obtain specific direct PCR amplification, a new primer pair (alf-fB1 5′-AACCCCT CAAAAGGTTTTAG−3′ and alf-rASHYS 5′-TG ATGATTTTTATTTATTATC−3′) was designed by modification of the original fB1 and rASHYS oligonucleotides, according to the ArAWB nucleotide sequence. The PCR conditions consisted in 35 cycles of: 1 min at 94 °C denaturation (3 min for the first step), 1 min 58 °C annealing and 2 min at 72 °C elongation. Total DNA from *Catharanthus roseus* infected with aster yellows phytoplasma (Torres et al., 2004), *Melia azedarach* infected with X-disease phytoplasma (Galdeano et al., 2004) and healthy alfalfa plants were used as negative controls. PCR products were analysed by electrophoresis through 1% agarose gel, stained with ethidium bromide and visualised using a UV transilluminator.

RFLP analysis

Ten μl of each 1.2 kb PCR amplified product, primed by R16F2/R16R2, from ArAWB and AshY1^T were individually digested with 16 restriction enzymes: *Alu*I, *Bam*HI, *Hha*I, *Hae*III, *Rsa*I, *Hinf* I, *Mse*I, *Eco*RI, *Kpn*I, *Sau*3AI, *Taq*I and *Pvu*II (New England Biolabs Inc., Beverly, MA, USA); *Dra*I, *Tha*I (Gibco, Life Technologies Inc., Maryland, USA); *Hpa*I and *Hpa*II (Promega). Manufacturers' instructions were followed in all cases. The 1.8 kb amplified fragments, obtained through P1/P7 primer amplifications, were digested with *Alu*I, *Hha*I, *Hinf*I, *Rsa*I and *Taq*I (New England Biolabs Inc). The digested products were electrophoresed on 1.5% agarose (Promega) plus 0.5% Metaphor agarose (Cambrex, NJ, USA) gel in TBE buffer and visualised as mentioned above. The RFLP patterns of the ArAWB phytoplasma were compared with those of AshY1^T reference strain and other phytoplasmas previously published (Lee et al., 1998; Griffiths et al., 1999; Barros et al., 2002). The ArAWB phytoplasma was classified into 16Sr groups according to Lee et al. (1998).

Cloning of PCR products and DNA sequencing

P1/P7 amplimers, obtained using the high fidelity synthesis *Pfu* DNA polymerase (Promega), were cloned into the multiple cloning site of pBluescript II SK + vector (Stratagene, CA, USA). Competent *Escherichia coli* DH5α strain cells were transformed with the recombinant plasmids following the Sambrook et al. (1989) procedure. Subclones of progressive unidirectional deletions, obtained with the Erase a Base System (Promega), were used for automatic sequencing. The 1.8 kb sequence of the rDNA sequence from ArAWB phytoplasma was recovered after a minimum of 2× sequencing coverage for each base position by overlapping. The sequences were assembled using the SeqMan programme (Lasergene software DNASTAR Ver. 5, 2001), and manual adjustment was done when necessary. The nucleotide sequence was deposited in the GenBank data library (Accession No. AY147038).

Sequence analysis

The 16S rRNA gene sequence of the ArAWB phytoplasma was compared with those of group 16Sr VII (EriWB, AshY1, Ashy3, Ashy5 and LWB3) and other closely related phytoplasmas (BLL, EY1 and ULW) (see Figure 4 for accession numbers). Almost full 16S rRNA gene and 16S–23S spacer region nucleotide sequences were aligned using the ClustalW option of the MegAlign programme (Lasergene software DNASTAR Ver. 5, 2001) and sequence homology was obtained. The similarity coefficients (F) among the

ArAWB and the phytoplasmas mentioned above were calculated according to Nei and Li (1979). Since DNA from all the analysed phytoplasmas was not available, putative restriction fragment analysis was performed from the partial sequences of the 16S rRNA gene (between primers R16F2 and R16R2) using the MapDraw programme (Lasergene software DNASTAR Ver. 5, 2001) for the 17 enzymes used for general classification (Lee et al., 1998). The *Alu*I, *Hha*I, *Hinf*I, *Taq*I and *Pvu*II restriction profiles obtained with P1/P7 primers from the ArAWB and AshY1^T were putatively compared with AshY1, AshY3, AshY5, LWB3 and EriWB phytoplasma patterns. The similarities and differences among the RFLP patterns of the ArAWB and the other phytoplasmas from group 16Sr VII rDNA were established by comparing the profiles defined by Griffiths et al. (1999), and the putative patterns based on the rDNA sequence of the EriWB phytoplasma (Barros et al., 2002).

Phylogenetic analysis

The phylogenetic tree was constructed by parsimony analysis with PHYLIP (Phylogeny Inference Package) Version 3.57c by Felsenstein (1993). The ArAWB DNA sequence was compared with almost full 16S rDNA and 16S–23S spacer region sequences of five AshY strains, seven closely related phytoplasmas, and *Acholeplasma palmae* as the out-group to root the tree. Bootstrapping was performed 1000 times to estimate the support of the inferred clades (Felsenstein, 1987). The consensus tree was drawn using the Tree View Programme (v1.6.5; by Page, 1996).

Results

Symptoms in diseased alfalfa plants

Infected alfalfa plants in the field showed symptom gradation ranging from plants with severe stunting, leaf size reduction, shoot proliferation and flower abnormalities (Figure 1) to almost asymptomatic. Ultrathin cross-sections of leaf midrib symptomatic plants revealed abundant pleomorphic bodies in the phloem tissue while no bodies were observed in the sieve tubes of the examined asymptomatic plants. Experimental transmission was successfully performed in alfalfa plants by

Figure 1. Symptomatic alfalfa plant (*Medicago sativa*) affected by ArAWB phytoplasma. Severely reduced leaf size as well as shoot shortness can be seen in diseased plants.

grafting infected scions to healthy plants grown in a greenhouse. Two months after grafting, symptoms similar to the ones observed in the field became evident. A colour variation from normal dark green in healthy plants to light green in infected plants was also observed.

PCR amplification

Fifty symptomatic alfalfa plants, collected from two different areas (Pocito and San Martín) were tested, and positive PCR results were obtained in all cases. From each sampling area, one PCR positive sample was selected to perform the PCR-RFLP analysis. Fragments of 1.2 and 1.8 kb length were obtained by PCR from both isolates and from AshY1^T reference strain DNA, using universal primers R16F2/R16R2 and P1/P7, respectively. PCR amplification was obtained from infected alfalfa samples with the specific AshY1 fB1/rAshYs primers only by nested PCR. Instead, all symptomatic alfalfa plants resulted positive when direct PCR was performed with the modified alf-fB1/alf-rASHYS primer pair (data not shown). PCR amplifications of 1.5 kb were obtained from alfalfa diseased plants but not from the AshY1^T, aster yellows and western X reference phytoplasmas.

RFLP analysis

*Rsa*I, *Dra*I, *Hpa*I, *Hpa*II, *Bam*HI, *Tha*I, *Hae*III, *EcoR*I, *Sau3*AI and *Kpn*I endonucleases digestions from the 1.2 kb ArAWB fragment showed the same profiles as those of the AshY1^T reference strain, described by Lee et al. (1998). However, differences were observed for *Alu*I, *Mse*I, *Taq*I, *Hha*I and *Hinf*I digestion patterns. Due to the small size of the differential fragments that resulted from the *Alu*I and *Mse*I digestions, higher resolution polyacrylamide gels were used in such cases (data not shown). The ArAWB *Alu*I pattern was identical to the one described for the EriWB (Barros et al., 2002) and the clover phyllody group, confirming that this enzyme pattern is not exclusive for the ash yellows group (Lee et al., 1998). *Hha*I, *Hinf*I and *Taq*I endonucleases showed unique profiles for ArAWB when compared to EriWB or other AshY strains. In addition, a unique *Pvu*II profile was observed in ArAWB. The *Rsa*I pattern for the 1.8 kb fragment digestions in ArAWB remained similar to AshY1^T; however, differences were evident in *Alu*I, *Hinf*I, *Hha*I and *Taq*I restriction profiles (Figure 2). No differences were observed between Pocito and San Martín ArAWB isolates based on collective RFLP patterns.

Sequence analyses

The sequence homology among almost full 16S rDNA and 16S–23S spacer region of ArAWB and AshY strains ranged from 97.7% (LWB3) to 98.8% (EriWB). The homology with the most closely related phytoplasma from a different group (BLL,

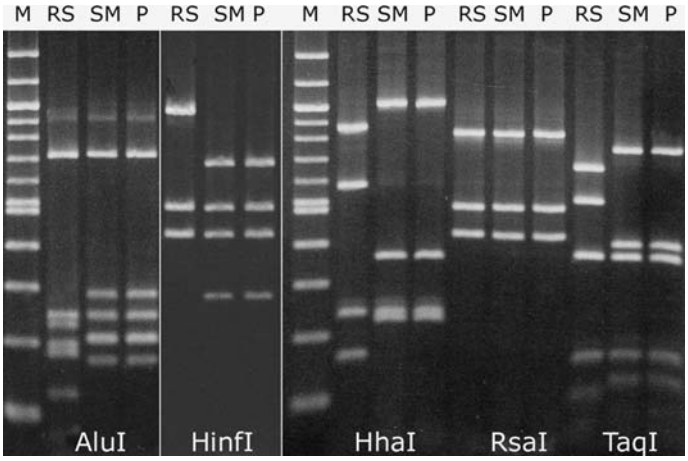

Figure 2. RFLP analysis of 1.8-kb PCR product (P1/P7 primers) of AshY1 Reference Strain (RS), San Martín ArAWB isolate (SM) and Pocito ArAWB isolate (P) were digested with *Alu*I, *Hinf*I, *Hha*I, *Rsa*I, and *Taq*I restriction enzymes. M: 100 bp DNA ladder (New England Biolabs Inc.) (100, 200, 300, 400, 500, 517, 600, 700, 800, 900, 1000, 1200 and 1517 bp).

16Sr VI clover proliferation group) was 96.9% (Table 1). The two ash yellows phytoplasma signature sequences, descriptive of '*Ca.* Phytoplasma fraxini' 5′-CGGAAACCCCTCAAAAGGTTT−3′ and 5′-AGGAAAGTC−3′ (Griffiths et al., 1999), were found in the 16S rDNA of ArAWB. However, those regions where alf-fB1/alf-rASHYS primer pair were designed had seven different nucleotides, two insertions and three deletions, when compared with the original fB1/rASHYS Ash Y group specific primer pair (Smart et al., 1996).

Putative restriction site analysis and similarity coefficients

The putative restriction site patterns defined from the ArAWB sequence analysis were coincident with those obtained by RFLP with the 16 enzymes assayed. The similarity coefficients calculated from the putative RFLPs of the 16S rDNA 1.2 kb fragment are shown in Table 2. The coefficients among the ArAWB phytoplasma and strains of the 16SrVII group ranged from 0.86 (with AshY3 and LWB3) to 0.92 (with EriWB). When the ArAWB sequence was compared with members of closely related groups, the similarity coefficients obtained were 0.88 for group 16SrVI (BLL phytoplasma) and 0.83 for group 16SrV (EY1 phytoplasma). The putative restriction patterns of the ArAWB almost full 16S rDNA and 16S–23S spacer region, generated by five endonucleases, was compared with those of group 16SrVII phytoplasmas (AshY1, AshY3, AshY5, LWB3, EriWB) (Figure 3). A *Hinf*I recognition site found in the ArAWB sequence (position 609) was absent in all the other ash yellows group phytoplasmas. Moreover, this pattern was shown to be exclusive not only within the AshY group but also among the described phytoplasmas (Lee et al., 1998). Several changes were observed in the *Mse*I restriction sites. ArAWB phytoplasma, like the EriWB, showed differences with the other group members in four *Mse*I sites (positions 1067, 1096, 1706 and 1718). Besides, two new *Mse*I sites (positions 1539 and 1547), unique for the ArAWB phytoplasma, were found. The *Alu*I site at position 193 was present in all AshY strains but not in ArAWB and EriWB phytoplasmas while the *Alu*I site 1714 was absent only in ArAWB. Similarly to EriWB, AshY5 and AshY3 strains, ArAWB lacked one *Alu*I site at position 1390. Besides, a new *Alu*I site was detected only in ArAWB at position 1413. Two new unique *Taq*I sites were found at positions 1378 and 1398 in ArAWB whereas a *Taq*I site at position 264 was present in all the AshY strains but not in the alfalfa phytoplasma. Unlike other AshY strains, ArAWB did not have an *Hha*I site at position 1054. The putative restriction site analysis of the ArAWB phytoplasma sequence determined unique patterns for enzymes *Alu*I, *Hha*I, *Hinf*I, *Taq*I and *Mse*I. On the basis of these data, Argentinean alfalfa witches′-broom phytoplasma is included into group 16SrVII although it clearly differs from the described AshY strains.

Phylogenetic analysis

The phylogenetic analysis of nearly full-length 16S rDNA sequence and 16S–23S spacer region from 13 diverse phytoplasmas, including Argentinean alfalfa witches′-broom phytoplasma and *Acholeplasma palmae* as out-group yielded a consensus tree (Figure 4). The bootstrapping values strongly

Table 1. Sequence homology among 16S rDNA from ArAWB phytoplasma and strains from groups 16SrVII (EriWB, Ash1, Ash3, Ash5, LWB); 16SrV (EY1, ULW) and unidentified mollicute (BLL)

Phytoplasma	ArAWB	EriWB	AshY1	AshY3	AshY5	LWB3	BLL	EY1	ULW
ArAWB	***	98.8	98.0	98.0	98.1	97.7	96.9	96.6	96.2
EriWB		***	98.5	98.6	98.6	98.2	96.9	96.4	96.1
AshY1			***	99.8	99.9	99.7	96.8	96.2	95.9
AshY3				***	99.9	99.5	96.7	96.3	96.0
AshY5					***	99.7	96.7	96.3	96.0
LWB3						***	96.5	96.0	95.6
BLL							***	96.4	96.0
EY1								***	99.4
ULW									***

Table 2. Similarity coefficients derived from putative RFLP analysis of 16S rDNA of ArAWB phytoplasma and the most closely related phytoplasmas belonging to groups 16SrVII (EriWB, Ash1, Ash3, Ash5, LWB); 16SrV (EY1, ULW) and unidentified mollicute (BLL)

Phytoplasma	ArAWB	EriWB	AshY1	AshY3	AshY5	LWB3	BLL	EY1	ULW
ArAWB	***								
EriWB	0.92	***							
AshY1	0.87	0.92	***						
AshY3	0.86	0.94	0.98	***					
AshY5	0.87	0.92	1	0.98	***				
LWB3	0.86	0.9	0.98	0.95	0.98	***			
BLL	0.88	0.89	0.86	0.87	0.86	0.85	***		
EY1	0.83	0.84	0.81	0.82	0.81	0.80	0.92	***	
ULW	0.80	0.82	0.79	0.8	0.79	0.78	0.89	0.97	***

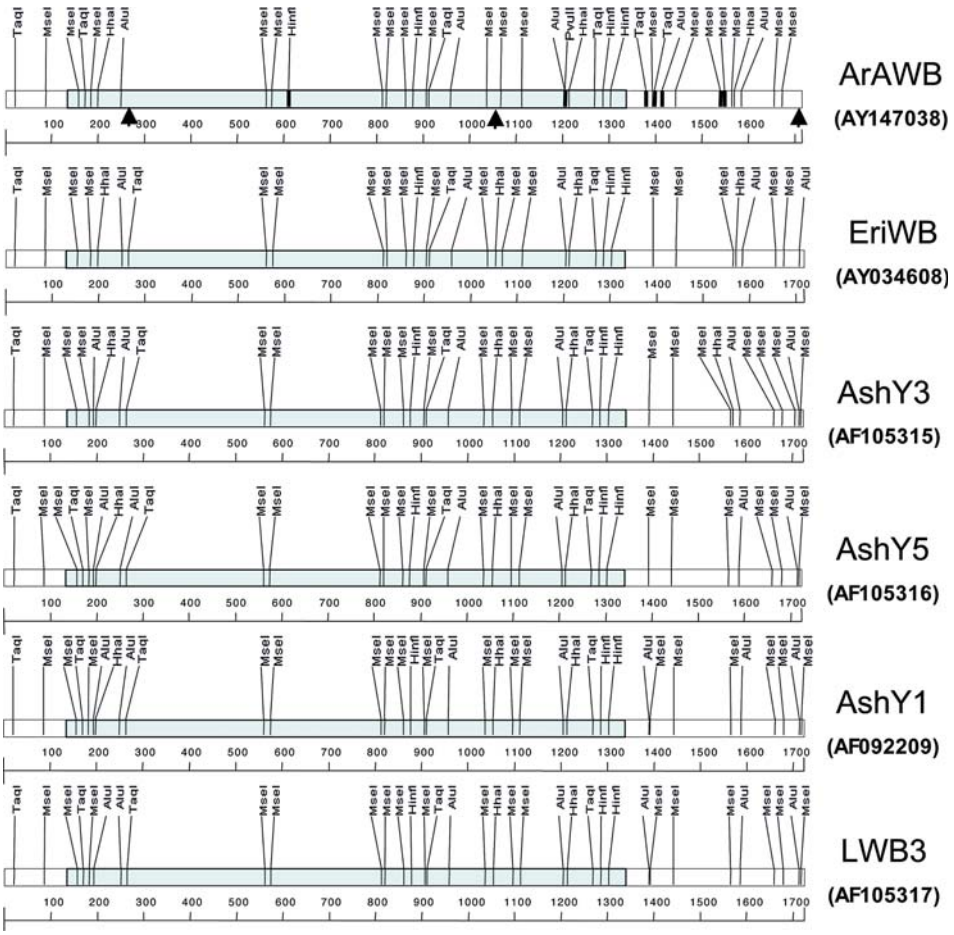

Figure 3. Putative restriction sites analysis in rRNA operon sequences from phytoplasmas belonging to group 16SrVII (Ash phytoplasma group) with cloned DNA sequence from ArAWB phytoplasma. Maps were generated using the MapDraw programme (DNASTAR package) for comparison of restriction endonucleases sites. Solid bar represents the 16S rDNA segment amplified by PCR with R16F2/R16R2 primers. Arrows below ArAWB map indicate lost restriction sites and solid lines show unique restriction sites in it. These changes differentiate ArAWB from all Ash phytoplasma strains. GenBank accession numbers of the ArAWB, EriWB (erigeron witches′-broom), AshY3 (ash yellows 3); AshY5 (ash yellows 5), AshY1 (ash yellows 1) and LWB3 (lilac witches′-broom) phytoplasma DNAs are in parenthesis.

supported most branches, and the branching order generally agreed with previously reported trees (Lee et al., 1998; Seemüller et al., 1998; Griffiths et al., 1999; Barros et al., 2002). The phytoplasma associated with alfalfa witches′-broom disease was included into the 16SrVII (ash yellows) group clade, generating a new branch (988 bootstrap value) that separated it from the AshY strains and EriWB.

Discussion

In this study we report an extended characterisation of an ash yellows phytoplasma affecting alfalfa crops in Argentina which was associated with witches′-broom symptoms (Meneguzzi et al., 2001). The ArAWB phytoplasma was detected by PCR with universal and specific primers and characterised by 16S rDNA RFLP and sequence analysis. Based on the unique RFLP patterns and significant differences found in the ArAWB 16S rRNA gene sequence in comparison with the previously described ash yellows strains (Griffiths et al., 1999; Barros et al., 2002), we propose it as a new 16SrVII subgroup. According to the profiles described by Griffiths et al. (1999) for the ash yellows 16S rDNA, the ArAWB showed distinct profiles after restriction with four endonucleases

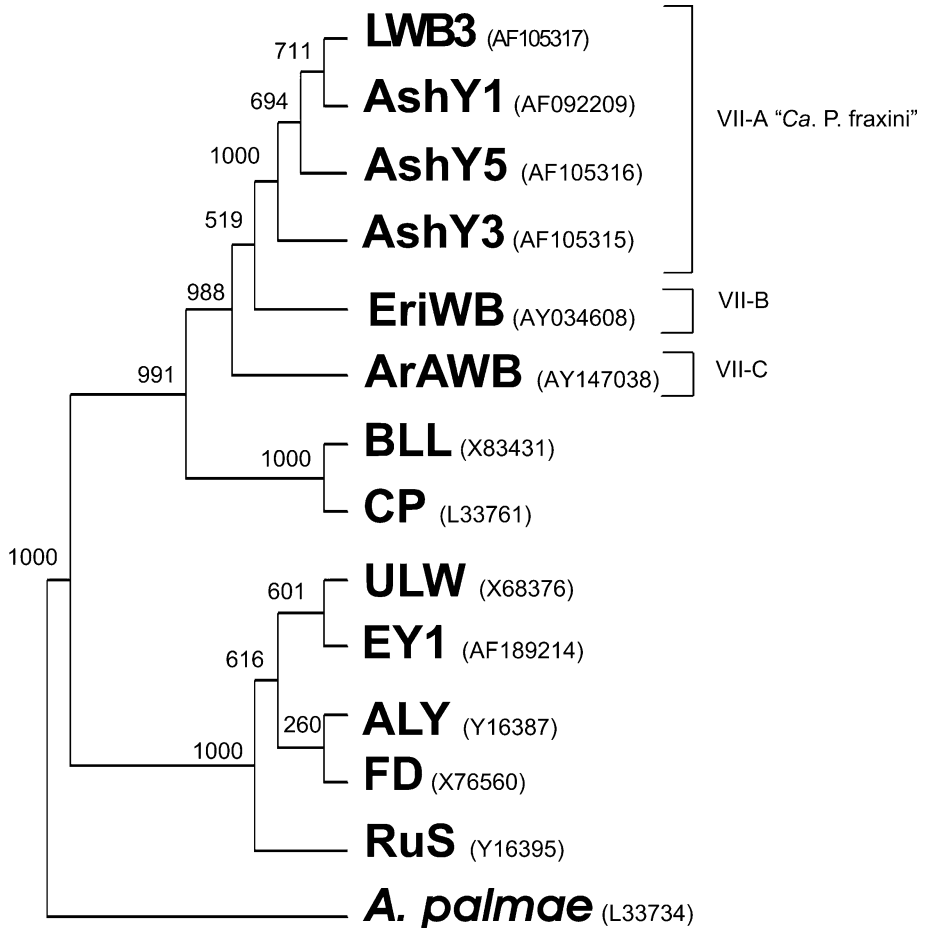

Figure 4. Phylogenetic tree constructed by parsimony of 16S rRNA gene sequences from 13 phytoplasmas and *Acholeplasma palmae* as the outgroup. The numbers on the branches are bootstrap (confidence) values. The strains used in the analysis are the following: '*Ca.* P. fraxini' LWB3 (Lilac witches′-broom;); '*Ca.* P. fraxini' AshY1 (Ash yellows 1); '*Ca.* P. fraxini' AshY3 (Ash yellows 3); '*Ca.* P. fraxini'' AshY5 (Ash yellows 5); ArAWB (argentinean alfalfa witches′-broom); EriWB (erigeron witches′-broom); BLL (brinjal little leaf); CP (clover proliferation); EY1 (elm yellows;); ULW (elm yellows); FD (flavescence doree); ALY (alder yellows) and RuS (rubus stunt).

used to differentiate the AshY strains. The sequence homology and similarity coefficient values derived from putative restriction site analysis were lower than those among subgroups A and B strains, and the phylogenetic analysis from the 16S rRNA confirmed the separation of ArAWB as a new subgroup within the ash yellows group. Based on the differences found we propose to establish subgroup 16SrVII-C to separate ArAWB from subgroup A and B.

In south America, progress has been done during the last years on the detection and classification of phytoplasmas associated with plant diseases. Phytoplasmas from groups 16SrI (aster yellows) (Torres et al., 2004), 16SrII (peanut witches′-broom) (Barros et al., 1998), 16SrIII (X-disease) (Barros et al., 1998; Conci et al., 1998; Harrison et al., 2003; Galdeano et al., 2004), 16SrIX (pigeon pea witches′-broom) (Barros et al., 1998), 16SrXIII (Mexican periwinkle virescence) (Harrison et al., 2003) and 16SrXV (hibiscus witches′-broom) (Montano et al., 2001), have been characterised. Besides the ArAWB phytoplasma, two phytoplasmas from the ash yellows group (16SrVII) have been reported in Colombia (Griffiths et al., 2001) and Brazil (Barros et al., 2002) infecting *Fraxinus chinensis* and *Erigeron* sp., respectively. The erigeron witches'-broom phytoplasma has been taxonomically located into a new subgroup B of the 16SrVII group (Barros et al., 2002) based on the differences found in the 16Sr DNA sequence with respect to the ash yellows strains described (Griffiths et al., 1999). The ArAWB phytoplasma 16S rRNA sequence contains the signature sequences for the proposed '*Ca.* Phytoplasma fraxini' (Griffiths et al., 1999) and the sequence similarities to the 16SrVII-A strains to include the ArAWB organism into a *Ca.* Phytoplasma species taxon (IRPCM Phytoplasma/Spiroplasma Working Team Phytoplasma Taxonomy Group, 2004). Further investigation related to the description of the vectors that transmit the ArAWB phytoplasma, the geographical distribution of '*Ca.* Phytoplasma fraxini' and additional molecular analysis will be necessary to confirm such an assumption.

The phytoplasmas that occur in south America can be included in the generally accepted phytoplasmas classification system; nevertheless, there have been cases in which the organisms did not fit any of the subgroups delineated. The hibiscus witches′-broom phytoplasma from Brazil has now been described as a new 16SrXV group and proposed as '*Ca.* Phytoplasma brasiliense' (Montano et al., 2001). Also a new subgroup for group 16SrIII (X-disease) has been proposed by Montano et al. (2000) for chayote witches′-broom phytoplasma in Brazil. Furthermore new hosts were described for phytoplasmas belonging to such a subgroup in Bolivia and Argentina (Harrison et al., 2003; Galdeano et al., 2004). The Argentinean alfalfa witches′-broom phytoplasma reported in this work, and the erigeron witches′-broom phytoplasma from Brazil (Barros et al., 2002), represent new subgroups of the ash yellows clade. It would be interesting to find out the relationship of these two phytoplasmas with the one detected in *Fraxinus chinensis* in Colombia in order to broaden the knowledge of the south American ash yellows group diversity. Interestingly both EriWB and ArAWB phytoplasmas were detected in herbaceous plants, in contrast to the ones found in *Fraxinus* and *Syringa*. Alfalfa crop was introduced into Argentina from México and Perú during the XVI century. Although no native plants, weeds or other crops have been found harbouring the ArAWB phytoplasma in Argentina, there is no evidence to infer that the disease could have been introduced with the crop. No reports were known about witches′-broom disease in alfalfa in Argentina until recent years. The phytoplasma seed transmission is hardly probable, and no ash group phytoplasmas have been reported in alfalfa elsewhere. Besides, the rDNA sequence of the ArAWB phytoplasma showed low homology when compared with ash yellows phytoplasmas found in north America. The high sequence similarity found between the ArAWB and EriWB phytoplasmas supports the idea that an evolutionary divergence could have occurred in south American phytoplasmas, originated by geographical or ecological separation (Montano et al., 2000, 2001); Barros et al., 2002.

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