ESTABLISHMENT OF THE NEW SUBGROUP 16SrI-S *(rr-rp) tuf*-H BELONGING TO '*Ca.* PHYTOPLASMA ASTERIS' IN WILD AND CULTIVATED PLANTS IN ARGENTINA

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SUMMARY

Argentinian catharanthus little leaf phytoplasma (ACLL) was found naturally infecting *Daucus carota* L. (carrot), *Catharanthus roseus* (LG Don) (periwinkle) and wild *Matricaria chamomilla* L. (chamomile). All infected plants showed symptoms of yellowing, little leaf and internodes shortening. Flower scape distortion, malformations of umbels and roots were observed in infected carrots and witches´broom in chamomile. Only periwinkles showed virescence and phyllody. On the basis of RFLP analyses of partial 16S rDNA, ribosomal protein rpl22 and *tuf* (EF-Tu) genes, ACLL phytoplasma was classified in group 16SrI ('*Ca.* Phytoplasma asteris'), new subgroup 16SrI-S *(rr-rp) tuf-H*. All ACLL phytoplasma strains have 16S rRNA interoperon sequence heterogeneity as shown by PCR-RFLP and nucleotide sequence analyses. Phylogenetic analysis using 16S rDNA sequences clustered ACLL phytoplasma strains into a new phylogenetic lineage within group 16SrI.

Key words: interoperon sequence heterogeneity, genetic polymorphism, sequencing, aster yellows subgroups, PCR-RFLP.

INTRODUCTION

Phytoplasmas ('*Candidatus* Phytoplasma' class *Mollicutes*) cause diseases in hundreds of economically important plants and are transmitted mainly by sap-feeding leafhoppers and psyllids (order Hemiptera) (Lee *et al.*, 2000; Weintraub and Beanland, 2006). Since phytoplasmas cannot be isolated in pure culture, their differentiation and classification rely on methods based on biophysical, biological or biochemical characters (Wei *et al.*, 2007).

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Restriction fragment length polymorphism (RFLP) and nucleotide sequence analysis of the rRNA genes have been useful for establishing a taxonomic scheme of phytoplasmas consisting of 16S rRNA groups and subgroups. Since the first comprehensive classification scheme was constructed based on RFLP analysis of PCR-amplified 16S rRNA (Lee *et al.*, 1993, 1998), the number of groups and subgroups has increased by the addition of new phytoplasmas. Wei *et al.* (2007) expanded the former classification scheme performing a computer-simulated RFLP analysis of 16S rRNA genes in which they included most of the available sequences. This scheme comprises 28 groups and more than 50 subgroups. More recently, an interactive online tool has been developed that generates and compares virtual RFLP profiles from the 16S rRNA gene sequences (Zhao *et al.*, 2009).

Because of the low level of polymorphism, the rDNA region is often not enough to distinguish closely related organisms (Botti and Bertaccini, 2003). Besides, heterogeneity in the 16S rRNA operon in a single genome can complicate classification, particularly by generating different RFLP patterns. However, many authors maintain that if minor differences exist between operons they should not be overestimated (Marcone *et al.*, 2000; Wei *et al.*, 2007). Therefore, other polymorphic markers, such as the elongation factor TU (EF-Tu; *tuf* gene) or the ribosomal protein operon (Gundersen *et al.*, 1996; Jomantiene *et al.*, 1998) have also been considered (Schneider *et al.*, 1997a; Marcone *et al.*, 2000). This has led to a better understanding of the great diversity and phylogenetic relationships of phytoplasmas (Lim and Sears, 1989; Namba *et al.*, 1993; Gundersen *et al.*, 1994, 1996; Seemüller *et al.*, 1998; Lee *et al.*, 1998, 2000; Marcone *et al.*, 2000; Jomantiene *et al.*, 2002; Wei *et al.*, 2007).

The 16SrI aster yellows phytoplasma group is relatively homogeneous at the rDNA level (Lee *et al.*, 1992, 1993; Gundersen *et al.*, 1996; Botti and Bertaccini, 2003), with no more than 2.6% nucleotide divergence between its members (Marcone *et al.*, 2000). The reference strain of the taxon '*Candidatus* Phytoplasma asteris' is the OAY phytoplasma, a member of subgroup

16SrI-B, *rp*I-B and *tuf*I-B that causes evening primrose (*Oenothera hookeri*) virescence (Lee *et al.*, 2004). This group presents the widest range of hosts geographical distribution (Lee *et al.*, 2004; Foissac and Wilson, 2010) and number of studied isolates in the world (Marcone *et al.*, 2000). In South and Central America, '*Ca.* Phytoplasma asteris'-related strains have been reported infecting basil, sweet pepper, broad bean, maize, sugarcane, grapevines (Arocha *et al.*, 1999, 2006, 2007; Bedendo *et al.*, 2000; Fiore *et al.*, 2007; Silva *et al.*, 2009).

In Argentina, 16SrI AY group phytoplasmas have a significantly lower prevalence than the 16SrIII (X-disease), 16SrVII (ash yellows) and 16SrXIII (Mexican periwinkle virescence) groups (Galdeano *et al.*, 2004, 2009; Arneodo *et al.*, 2005; Conci *et al.*, 2005; Meneguzzi *et al.*, 2008). In 2002, the maize bushy stunt phytoplasma (MBS, 16Sr I-B) was detected in different areas of Argentina, and the Argentinian catharanthus little leaf phytoplasma (ACLL, 16SrI), responsible for the little leaf symptoms in naturally infected periwinkle, was described in 2004 (Giménez Pecci *et al.*, 2002; Torres *et al.*, 2004a). A preliminary classification of the ACLL phytoplasma into the aster yellows 16SrI-B subgroup was determined by PCR-RFLP analysis of the 16S rRNA gene. In such work, although differences were found in the *Rsa*I and *Hae*III RFLP patterns, the 16S rRNA gene was considered insufficient to assign the ACLL phytoplasma to a new subgroup.

Recently, symptomatic plants of *Daucus carota* L. subsp. *sativus* (carrot), *Matricaria chamomilla* L. (chamomile) and periwinkle were collected in the central and north-eastern regions of Argentina. Samples of the three species tested positive for phytoplasmas by PCR and were classified by PCR-RFLP into the 16SrI aster yellows group (Torres *et al.*, 2004b). Interestingly, the three above species had been reported in the northern hemisphere as hosts of 16SrI aster yellows phytoplasmas of subgroups -A, B and C, (Davis *et al.*, 1990; Lee *et al.*, 1993; Khadhair *et al.*, 1999; Valiunas *et al.*, 2001; Valova *et al.*, 2002).

Thus, the objective of this study was to establish the taxonomic position and phylogenetic relations of the aster yellows phytoplasmas associated with witches' broom symptoms in chamomile, little leaf in periwinkle and proliferation and stunting in carrot.

MATERIALS AND METHODS

Plant samples and reference phytoplasma strains. Five isolates of ACLL phytoplasma were analyzed, three of which originated from periwinkles (ACLL*cba*, ACLL*jun* and ACLL*ctes*), one from carrot (ACLL*dau*) and the other from chamomile (ACLL*cham*). All the isolates came from the Córdoba province (central Argentina) except for ACLL*ctes* which was found in Corrientes (north-eastern Argentina).

All the plants exhibited yellowing, marked leaf size decrease and internode shortening. Other symptoms such as distortion of flower scapes, deformity of umbels and roots were observed in carrots, witches´ broom in chamomile, virescence and phyllody in periwinkles. Phytoplasma isolates were tranferred to isogenic periwinkle plants by grafting or by *Cuscuta subinclusa* and kept in a greenhouse.

American aster yellows (AAY) (provided by Dr. E. Seemüller) and maize bushy stunt (MBS) phytoplasmas (Gimenez Pecci *et al.*, 2002) were used as reference strains for subgroup 16SrI-B rpB.

PCR conditions and primers. Genomic DNA of symptomatic and symptomless plants (negative control) was recovered by CTAB extraction (Doyle and Doyle, 1990) and phytoplasmas were identified and characterized by PCR-RFLP analysis of rpl22, elongation factor TU and rRNA genes. Universal primers R16F2/R16R2 (Lee *et al.*, 1993) were used to amplify 1.2 kbp fragments from the 16S rRNA gene. Amplifications were performed in 40 µl reaction mixtures with 1 U Promega Taq DNA polymerase for each reaction as described (Lee *et al.*, 1993). Primers rpR1/rpF1 (Lim and Sears, 1992; Gundersen *et al.*, 1994) and f*Tuf*Ay/r*Tuf*Ay (Schneider *et al.*, 1997a) were used to amplify fragments of 1.2 kbp and 940 bp aproximately, which correspond to rpl22 and *tuf* (EF-Tu) genes, respectively.

PCR runs were done using a Biometra TRIO-Thermoblock, programmed under the following specific conditions: (i) primers R16F2/R16R2 and rpR1/rpF1, 35 cycles of 1 min at 94ºC (3 min for the first cycle), 2 min at 52ºC and 2 min at 72ºC, and 5 min at 72ºC for a final elongation; (ii) primers f*Tuf*Ay/r*Tuf*Ay, 35 cycles of 30 sec at 95ºC (2 min for the first cycle), 30 sec at 55ºC and 1 min at 72ºC, and 5 min at 72ºC for a final elongation. PCR products were analyzed by 1% agarose gel electrophoresis and ethidium bromide staining.

RFLP analysis of amplified 16S rRNA, *tuf* and rpl*22* genes. Amplified products of the 16S rDNA (primers R16F2/R16R2) were digested with restriction endonucleases *Alu*I, *Hba*I, *Hae*III, *Hpa*II, *Kpn*I, *Mse*I, *Rsa*I, *Sau3*AI, *Taq*I and *Hinf*I (New England Biolabs, USA) (Lee *et al.*, 1993, 1998). The rpl22 gene amplified fragments (primers rpR1/rpF1) were digested with *Alu*I, *Dra*I, *EcoR*I, *Hha*I, *Hinf*I, *Hpa*II (NEB) and *Tru*II, (isoesquizomere of *Mse*I enzyme) (Fermentas, Canada). The *tuf* gene fragments (primers f*Tuf*AY/r*Tuf*AY) were digested with *Sau3*AI, *Alu*I, *Hpa*II (New England Biolabs) according to Marcone *et al.* (2000). Digestions were done for 16 h in individual reactions of 20 µl using 150-200 ng of PCR product, following the manufacturers' recommendations for each enzyme. The products were separated into 1.5% agarose (Promega, USA) + 0.5% agarose MetaPhor gel, in TBE buffer (0.5X).

Polyacrylamide 6% gel was used when necessary to a better resolution of difficult-to-define patterns in agarose. RFLP profiles were compared with those obtained from reference DNAs, and with those previously published (Gundersen *et al.*, 1996; Lee *et al.*, 1998, 2004; Marcone *et al.*, 2000; Jomantiene *et al.*, 2002; Torres *et al.*, 2004b).

Cloning, nucleotide sequencing and putative restriction site analysis. 16S rDNA fragments of isolates ACLL*cba* amplified with R16F2/R16R2 primers, and ACLL*cham* and ACLL*ctes* amplified with P1/P7 primers (Deng and Hiruki, 1991; Schneider *et al.*, 1997b), were cloned and sequenced. PCR fragments were purified and treated with Klenow large fragment polymerase (Promega, USA) to generate blunt ends. The fragments were ligated into Bluescript II SK+ (Stratagene, USA) vector in the polylinker cloning site, digested with *Sma*I. Then, competent *Escherichia coli* DH5α cells were transformed. The clones obtained were sequenced with an automatic sequencer and the sequences deposited in the NCBI GenBank (http://www.ncbi.nlm.nih.gov)

The sequences were aligned and edited using the multiple sequence alignment option of the MEGA version 4 bio-computational software (Tamura *et al.*, 2007). 16S rRNA gene sequences of ACLL*ctes* and ACLL*cham* isolates and both operons of the ACLL*cba* isolate (rrnA and rrnB) were compared with 33 phytoplasmas sequences of subgroup 16SrI, including '*Candidatus* phytoplasma asteris' [16SrI-B, *rp*I-B and *tuf*I-B strains, Lee *et al.* (2004). Sequences of '*Ca.* phytoplasma lycopersici', '*Ca.* phytoplasma japonicum', '*Ca.* phytoplasma australiense', Stolbur (16Sr XII). The Mexican periwinkle group (16SrXIII) phytoplasmas, closely related to *Ca.* Phytoplasma asteris (Zhao *et al.*, 2010), were also included in the analysis. Sequences of *Acholeplasma palmae* and *A. laidlawi* were used as outgroup. A consensus cladogram (Tree Explorer option of Mega version 4 software) was generated to estimate the phylogenetic relationships between the ACLL isolates and the other aster yellows phytoplasmas. The analysis was based on the neighbour-joining method, with the reliability provided by 1000 replications bootstrap test.

The analysis of the putative restriction sites for *Rsa*I and *Hae*III enzymes, which had shown differences in the PCR-RFLP assays, was conducted in the 16S rDNA sequences of ACLL phytoplasma isolates, and in the sequences of 16Sr-I subgroups representatives BB (-A), AAY (-B), MBS (-B), *Ca.* phytoplasma asteris (OAY), OY (-B), CPh operon A (-C), CPh operon B (-C), PaWB (-D), BBS3 (-E), ACLR–AY (-F), STRAW2 (-K), AV2192 (-L), AVUT (-M), IOWB (-N), ChLL (-Q) and ScYL-Br, retrieved from GenBank, using the sequence analysis DNASTAR software (DNASTAR, USA) MapDraw option.

The ACLL phytoplasma isolates classification into 16Sr groups and subgroups was also established by the putative RFLP similarity coefficient analysis using the *i*PhyClassifier online program (Zhao *et al.*, 2009).

RESULTS

Phytoplasma detection and RFLP analysis of amplified 16S rRNA, *tuf* and rpl*22* genes. Phytoplasma presence was detected in symptomatic carrot, chamomile and periwinkle plants analyzed by PCR with primers R16F2/R16R2, while there was no amplification from DNA of healthy plants (not shown).

PCR-RFLP analysis of the three genomic regions (rRNA operon, *tuf* and rpl22 genes) showed that the ACLL phytoplasma isolates are closely related to each other and are not typical members of subgroup 16SrI-B. The 16S rRNA gene restriction patterns generated by *Alu*I, *Hha*I, *Hpa*II, *Kpn*I, *Mse*I, *Taq*I, *Hinf*I and *Sau*3AI were like those of the 16SrI-B reference phytoplasmas (AAY and MBS) and other 16SrI-B subgroup phytoplasmas (Lee *et al.*, 1998) (not shown). However, ACLL phytoplasma isolates had differential *Hae*III and *Rsa*I patterns. *Rsa*I is one of the three 'key' enzymes considered for the classification of group 16SrI (Wei *et al.*, 2007). The digestion with *Rsa*I generated the same electrophoretic profile for all the ACLL isolates, but different from those of AAY and MBS strains (Fig. 1) and other 16SrI-B phytoplasmas published by Lee *et al.* (1998). The new pattern consisted of three bands of 425, 337 and 266 bp, which are distinctive of subgroup B, and two additional bands of 210 and 110 bp. Digestion with *Hae*III generated a similar but not identical pattern to that of MBS with one additional band 151 bp in size, which was not present in AAY or other aster yellows phytoplasmas reported by Lee *et al.* (1998). The RFLP patterns generated by both enzymes were composed of DNA fragments that totalled a size larger than the non-digested PCR product, suggesting interoperon sequence heterogeneity (Fig. 1).

When the PCR-RFLP assay was performed with enzymes *Rsa*I and *Hae*III on the 16S rDNA cloned fragments of ACLL*cba*, two restriction patterns (I and II) were produced by each enzyme. When the patterns were superimposed, the profile obtained was similar to that of the fragment amplified directly from the infected plants (uncloned) DNA (Fig. 2). Class I pattern of clone ACLL*cba*-I characterizes one operon (rrnA) and class II pattern of clone ACLL*cba*-II characterizes the second operon (rrnB).

Amplification and RFLP analysis of *rp* gene operon and *tuf* gene phytoplasma. Fragments of the expected size (approximately 1.2 kbp and 940 bp) resulted from PCR reactions using primers rpF1/rpR1 and

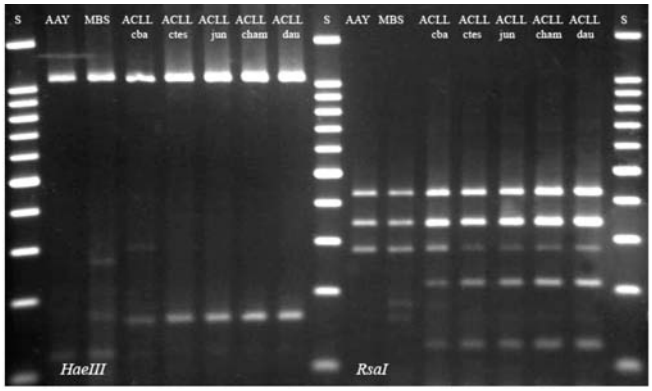

Fig. 1. RFLP analysis of 1.2kb PCR products (R16F2/R16R2 primers) of 16S rRNA gene digested with *Hae*III and *Rsa*I restriction enzymes. AAY: American aster yellows (control); MBS: Maize bushy stunt (control); ACLL*cba*, ACLL*ctes*, ACLL*jun*, ACLL*cham*, ACLL*dau*: Argentinian catharanthus little leaf isolates; S: 100 bp ladder (Promega), fragment sizes (bp) from top to bottom: 1500, 1000, 900, 800, 700, 600, 500, 400, 300, 200, 100.

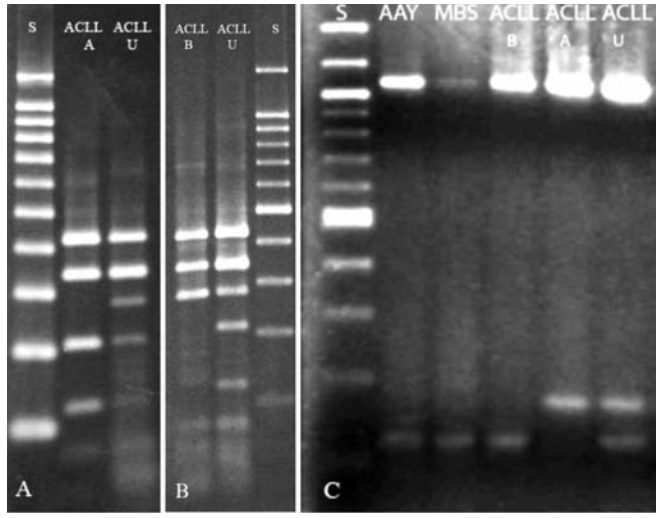

Fig. 2. *Rsa*I (A - B) and *Hae*III (C) RFLP analysis of cloned (ACLL rrn operons A and B) and infected uncloned (U) plant genomic DNA of Catharanthus little leaf phytoplasma, (ACLL*cba*). S, 100 bp ladder (Promega, USA).

f*Tuf*AY/r*Tuf*AY, respectively, from DNA of plants infected with isolates ACLL*cba*, ACLL*jun*, ACLL*ctes*, ACLL*dau* and ACLL*cham*.

The RFLP patterns of the *tuf* gene of ACLL isolates differed from those proposed by Marcone *et al.* (2000) as regards the digestion with *Alu*I (Fig. 3), while no differences were observed in the patterns corresponding to *Sau3*AI and *Hpa*II (not shown).

All the isolates presented the same patterns for the rpl*22* gene digested with *Tru*II and *Alu*I. *Tru*II profile was similar but not identical to that of AAY, and *Alu*I differed from the AAY and MBS patterns. The isolates of ACLL phytoplasma showed a restriction pattern for these enzymes previously unreported (Fig. 4).

Nucleotide sequences and putative restriction sites in 16S rRNA and phylogenetic analysis. The 16S rDNA gene of isolates ACLL*cba*, ACLL*ctes* and ACLL*cham* was cloned and sequenced. In the case of ACLL*cba*, two clones with different RFLP patterns were sequenced, corresponding to rrn operons A and B. The sequences were deposited in GenBank, i.e. ACLL*cba*I-rrnA, accession No. FN825680; ACLL*cba*II-rrnB, FN825681; ACLL*ctes*-rrnA, FN825682; ACLL*cham*-rrnB, FN825683. The putative restriction site maps (Fig. 5) were coherent with the PCR-RFLP profiles obtained from the uncloned DNA (Fig. 1). The differences observed in *Rsa*I and *Hae*III restriction sites corresponded to base substitutions. In rrnA operon (clones ACLL*cba*-I and ACLL*ctes*), substitutions at positions 266 and 110 on the sequences correlated well with the additional bands of 210 bp and 110 bp observed in the uncloned rDNA RFLP pattern generated by *Rsa*I. The change in site 107 explains the gain of one differential band of 151 bp in the *Hae*III uncloned rDNA RFLP patterns (Fig. 1). This allowed us to confirm that the ACLL phytoplasma has sequence heterogeneity between operons. Also, the substitutions found in the restriction sites of

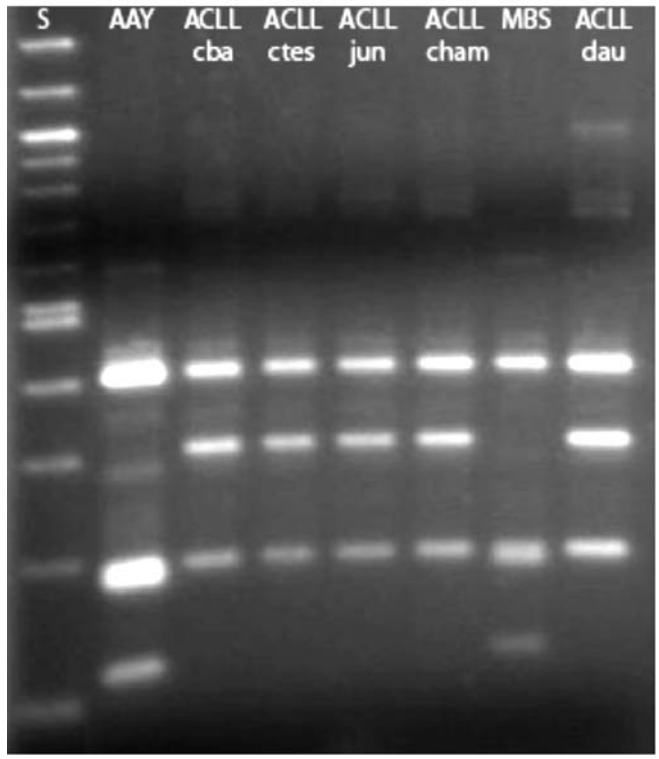

Fig. 3. *Alu*I restriction profile of a phytoplasma *tuf* gene fragment amplified with primer pair fTufAy/rTufAy. Phytoplasma strain abreviations: AAY (American aster yellows); ACLL*cba*, ACLL*ctes* ACLL*jun*, ACLL*cham* and ACLL*dau* (Argentinian catharanthus little leaf isolates); MBS (Maize bushy stunt); S, 100 bp ladder.

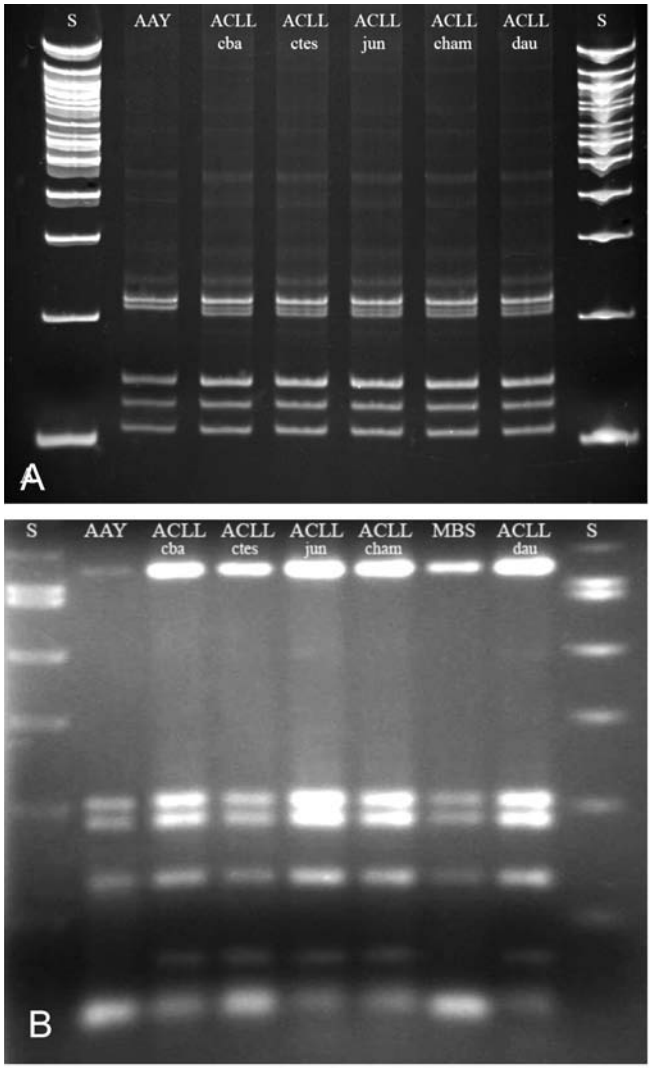

Fig. 4. RFLP analysis of rpl22 ribosomal protein gene operon sequences amplified by PCR with primers rpR1/rpF1 from phytoplasma strain AAY (American aster yellows); ACLL*cba* ACLL*ctes*, ACLL*jun*, ACLL*cham* and ACLL*dau* (Argentinian catharanthus little leaf isolates); MBS (Maize bushy stunt). The DNA products were digested with restriction enzymes *Tru*II (A) and *Alu*I (B). S, 100 bp ladder.

*Rsa*I and *Hae*III were clearly visualized when comparing the putative restriction site maps of the 16S rDNA partial sequence of ACLL*cba* (rrnA and rrnB), ACLL*ctes* (rrnA) and ACLL*cham* (rrnB) phytoplasmas with those of the '*Ca.* Phytoplasma asteris'-related strains OAY, AAY (Fig. 5), and with those of MBS, OY, BBS3, PaWB, ACLR-AY, BB, STRAW2, AV2192, AVUT, IOWB, CPh operon A, CPh operon B, ScYL-Br (not shown).

In agreement with these results, the online *i*PhyClassifier program (Zhao *et al.*, 2009) used to classify the phytoplasmas based on the comparison of putative RFLP similarity coefficient, showed that the ACLL phytoplasma is related to the 16SrI group, but different from all the known subgroups. The higher similarity coefficient (0.63) corresponded to 16Sr group I, subgroup B, suggesting that it should be considered as a new subgroup.

In order to estimate the phylogenetic relationship of the ACLL phytoplasma isolates with other members of the group 16SrI-AY, a multiple alignment was performed of the 16S rRNA gene partial sequence of the isolates ACLL*cba* (rrnA and rrnB), ACLL*cham* and ACLL*ctes* with 40 sequences from the GenBank (Fig. 6). The sequence similarity between the operons rrnA and rrnB of isolate ACLL*cba* was 99.5%, ACLL*cba* rrnA showed 99.6% similarity with ACLL*ctes* and ACLL*cham* while ACLL*cba* rrnB had 99.4% with ACLL*ctes* and 99.8% with ACLL*cham*. The sequences of ACLL*cba* operons rrnA and rrnB, ACLL*ctes* and ACLL*cham*, had 99.2%, 99.6%, 99.5%, and 99.7% similarity, respectively, when compared to '*Ca.* phytoplasma asteris'-related strain OAY. In general, the topology of the consensus tree agreed with others previously published (Valiunas *et al.*, 2009). The order of the branches showed a common origin for the ACLL isolates diverging from other phytoplasmas of the AY group.

DISCUSSION

The Argentinian catharanthus little leaf (ACLL) phytoplasma was found naturally infecting plants of *D. carota*, *M. chamomilla* and *C. roseus* from central and northeastern Argentina. In a previous work, the ACLL phytoplasma had been found in *C. roseus* plants and classified as a member of the aster yellows group (16SrI) by PCR-RFLP analysis of the 16S rDNA. The phytoplasma was related to subgroup 16SrI-B although the RFLP patterns were not identical to the reference strains, suggesting that the analysis of the rDNA was not enough for the ultimate establishement of phytoplasma affiliation (Torres *et al.*, 2004a). In this work we present evidence to include five isolates of ACLL phytoplasma (ACLL*cba*, ACLL*ctes*, ACLL*dau*, ACLL*jun* and ACLL*cham*) within a new subgroup 16SrI-S. These organisms are genetically homogenous and can be distinguished from other AY phytoplasmas by the RFLP profiles of 16S rRNA, *rp* and *tuf* genes.

RFLP analysis of the rRNA, *tuf* and rpl22 genes of ACLL isolates showed that, according to the current phytoplasma classification scheme (Lee *et al.*, 1998; Gundersen *et al.*, 1996; Jomantiene *et al.*, 1998; Botti and Bertaccini, 2003; Marcone *et al.*, 2000), they belong to group 16SrI (aster yellows) but present unique characteristics that clearly differentiate them from the reference strains of all the subgroups described.

The combined analysis of the RFLP collective profiles of the 16S rRNA operon, elongation factor TU (*tuf* gene) and ribosomal protein (rpl22 gene) revealed polymor-

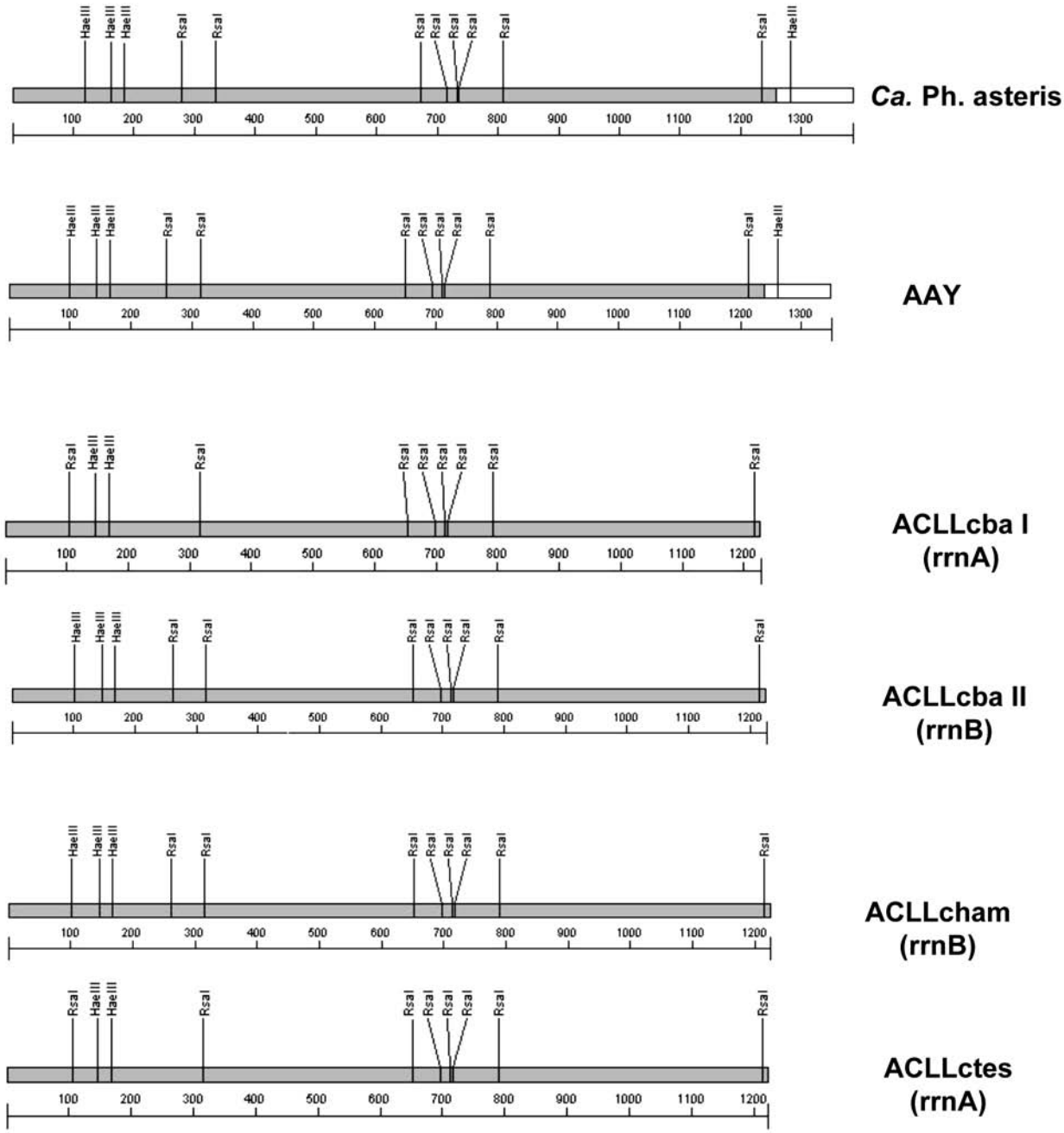

Fig. 5. Putative restriction sites in phytoplasma 16S rRNA gene sequences. Maps were generated by using the MapDraw options of the DNASTAR program (DNASTAR, Inc.) for comparison of recognition sites for the restriction endonucleases *Rsa*I and *Hae*III. Arrows indicate different putative restriction sites between operons.

phism with unique patterns generated by *Rsa*I, *Hae*III, *Alu*I and *Tru*II. Enzyme *Rsa*I, as well as *Mse*I and *Hinf*I, is of particular interest as a molecular marker since it is considered 'key' for phytoplasmas classification at the group level based on '*in silico*' generated RFLP of the 16S rRNA gene (Wei *et al.*, 2007). All ACLL phytoplasma isolates from different plant species and geographical regions showed identical *Rsa*I and *Hae*III patterns, made up of supernumerary bands, which are coincident with the addition of operon rrnA and rrnB patterns, which can be taken as evidence of ribosomal gene operon sequence heterogeneity. Many phytoplasmas from this and other 16Sr groups were reported to have heterogenous rRNA operons (Marcone *et al.*, 2000; Jomantiene *et al.*, 2002; Liefting *et al.*, 1996).

The sequence of the elongation factor TU (*tuf* gene) is more variable than that of the 16S rRNA gene, thus more useful for differentiating and classifying closely related organisms (Schneider *et al.*, 1997a). The *tuf* gene of all ACLL isolates has a unique *Alu*I RFLP pattern. Therefore, following Marcone *et al.* (2000) criterion, a new *tuf*-H pattern can be defined based on the ACLL

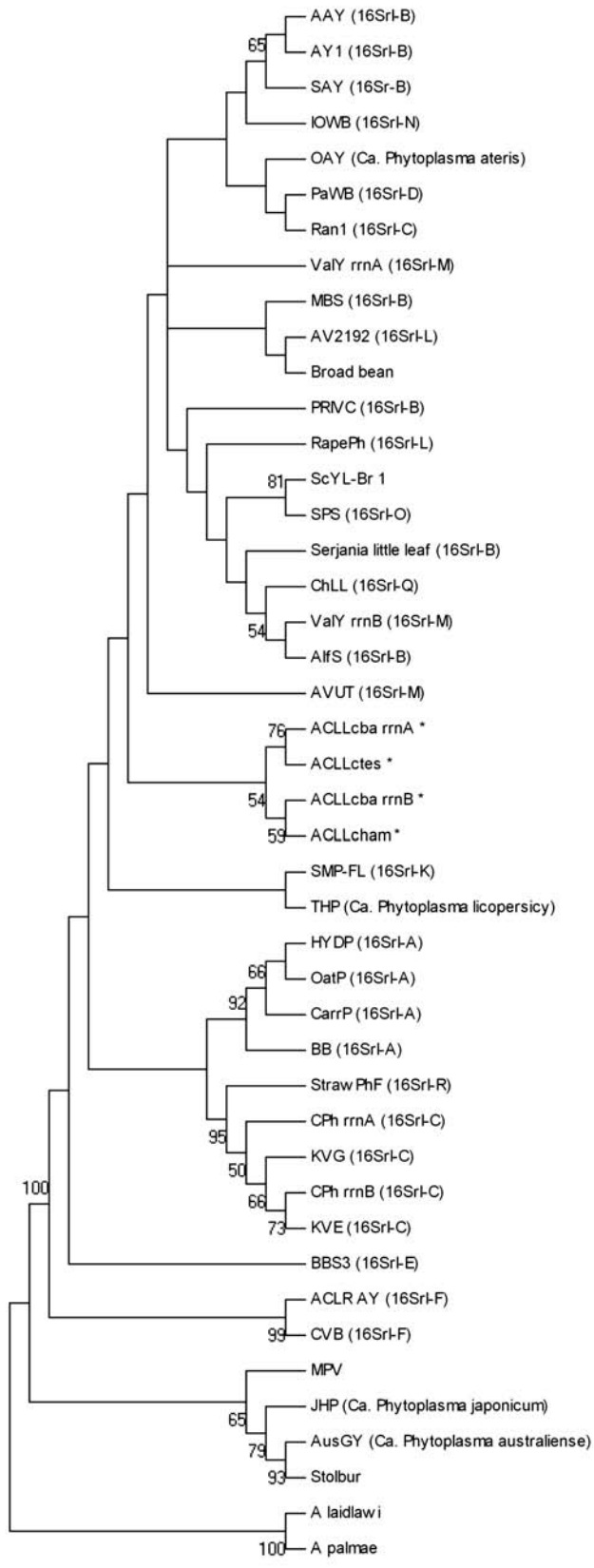

Fig. 6. Phylogenetic tree constructed by the neighbour-joining method of 16S rRNA gene sequences from 33 phytoplasmas 16SrI-aster yellows representative subgroups strains, other related 16Sr phytoplasmas groups and *A. palmae* and *A. laidlawi* as outgroup. The numbers on the branches are bootstrap (confidence) values. GenBank accessions numbers of phytoplasma 16S rRNA gene sequences: AAY (American aster yellows) X68373; AY1 (Maryland aster yellows) AF322644; SAY (Severe aster yellows) AF222063; IOWB (Ipomoea obscura witches'-broom) AY265205; OAY (*Ca*. Phytoplasma asteris) M30790; PaWB (Paulownia witches'-broom) AY265206; Ran1 (Ranunculus phyllody) AM990989; ValY (Valeriana yellows, rrnA) AY102274; MBS (Maize bushy stunt) AY265208; AV2192 (Aster yellows phytoplasma) AY180957; Broad bean, DQ286953; PRIVC (Primrose virescence) AY265210; RapePh (Rape phyllody) U89378; ScYL-Br1 (Sugarcane yellows leaf) EU423900; SPS (Soybean purple stem) AF268405; Serjania little leaf (AY725210); ChLL (Cherry little leaf) AY034089; ValY (Valeriana yellows, rrnB) AF177384; AlfS (Alfalfa stunt) AF177384; AVUT (Aster yellows phytoplasma) AY265209; SMP-FL (Strawberry multiplier) EF042898; THP (*Ca*. Phytoplasma lycopersici) EF199549; HyDP (Hydrangea phyllody) AY265215; OatP (Oat proliferation) AF453416; CarrP (Carrot proliferation) AF291682; BB (Tomato big bud) AY180955; StrawbPhF (Strawberry phyllloid fruit) AY102275; CPh (Clover phyllody, rrnA) AF222065; KVG (Clover phyllody) X83870; CPh (Clover phyllody, rrnB) AF222066); KVE (Clover phyllody) AY265217; BBS3 (Blueberry stunt) AY265213; ACLR AY (Apricot chlorotic leaf roll) AY265211; CVB (Leafhopper-borne) AY265212; MPV (Mexican periwinkle virescence) AF248960; JHP (*Ca*. Phytoplasma japonicum) AB010425; AusGY (*Ca*. Phytoplasma australiense) L76865; Stolbur, AF248959; *A. laidlawi* (M23932); *A. palmae* (L33734). *: Strains ACLL sequenced in this study.

phytoplasma RFLP *tuf* gene. Gundersen *et al.* (1996) and Botti and Bertaccini (2003) observed that the ribosomal protein gene revealed variations among very closely related organisms, such as AY phytoplasmas belonging to subgroup 16SrI-B, whose differences are not detected in the ribosomal DNA region. The AAY strain has the rpl*22* gene typical pattern for subgroup -B as described by Botti and Bertaccini (2003). The MBS and IOWB phytoplamas, which are also members of subgroup 16SrI-B, have different rpl*22* gene RFLP patterns, for which Gundersen *et al*. (1996) proposed the new subgroups 16SrI-H (rr-rp) and 16SrI-F (rr-rp), respectively. Since the ACLL phytoplasma isolates present a unique RFLP pattern for the rpl22 gene, we define the new subgroup as 16SrI-S (rr-rp) based on the combined analysis of the 16Sr rRNA and rpl22 genes and following the classification criterion proposed by Botti and Bertaccini (2003), Jomantiene *et al.* (1998) and Lee *et al.* (1998). The rDNA partial sequence of the ACLL phytoplasma isolates showed high similarity (99.4%-99.8%) with phytoplasmas from subgroups 16SrI-B (MBS, ScYL-Br and AlfS), -C (Ran1), -M (Valeriana rrnA and AVUT), -N (IOWB) and -L (AV2192; Rape Ph). This result reinforces the necessity of analyzing more variable genomic regions to allow a better definition of phytoplasma taxonomic position (Marcone *et al.*, 2000; Gundersen *et al.*, 1996; Lee *et al.*, 1998, 2004).

The phylogenetic analysis of the 16S rDNA partial

sequence generated a tree similar to that constructed by Valiunas *et al.* (2009) and grouped ACLL phytoplasma isolates in a discrete clade, closely related to '*Ca.* Phytoplasma asteris'.

South American phytoplasmas can be included in the generally accepted phytoplasmas classification system although there have been cases in which the organisms did not fit any of the extant subgroups (Montano *et al.*, 2001; Galdeano *et al.*, 2004; Conci *et al.*, 2005; Arneodo *et al.*, 2007). The isolates of the ACLL phytoplasma delineate a phylogenetically divergent clade within the aster yellows group. Although they have high sequence similarity, when compared to other members of group 16SrI, they can be clearly differentiated by the RFLP analysis of 16S rRNA, ribosomal protein rpl22 and *tuf* genes. The results obtained show that, even with high similarity values between the nucleotide sequences, the RFLP analysis is a consistent tool for the identification, differentiation and classification of phytoplasmas since it detects differences in informative sites of a highly conserved genomic region, as stated by Valiunas *et al.* (2009).

The aster yellows phytoplasmas detected in carrot, chamomile and periwinkle in Argentina are isolates of the ACLL phytoplasma and differ from the OAY phytoplasma proposed as reference strain of the subgroup 16Sr-B, rpI-B (Lee *et al.*, 2004) and all the known 16SrI phytoplasmas. The unique characteristics of the ACLL phytoplasma within the 16SrI group reinforce the theory of an evolutionary divergence of South American phytoplasmas, originated by geographical or ecological separation (Montano *et al.*, 2001; Barros *et al.*, 2002).

Up to the present, no phytoplasmas have been reported infecting *M. chamomilla* or *D. carota* in the southern hemisphere. Chamomile has a broad geographical distribution and coexists with cultivated crops, representing a putative phytoplasma reservoir and a refuge for its vectors (Valova *et al.*, 2002). Besides the molecular analysis, other elements such as host range, distribution and vector transmission are to be taken into account in order to develop further studies on the management and control of these diseases.

ACKNOWLEDGEMENTS

This work was supported by PICT 08-15219 and PICT 08-12914 from Agencia Nacional de Promoción Científica y Técnica - Argentina, and INTA-AEPV 214012. F. Fernández holds a doctoral fellowship from FONCyT (grant 904). E. Galdeano is a researcher of the CONICET (Consejo Nacional de Investigaciones Científicas y Técnicas - Argentina). INTA (Instituto Nacional de Tecnología Agropecuaria). The authors very specially thank two anonymous reviewers for their comments, which have positively contributed to the improvement of the manuscript.

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Received August 23, 2010
Accepted February 25, 2011

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