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Genetic diversity of 16SrIII group phytoplasmas in Argentina. Predominance of subgroups 16SrIII-J and B and two new subgroups 16SrIII-W and X

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Abstract Twelve Argentinean 16SrIII (X-disease)-group phytoplasma strains were analyzed. Ten of them, detected in daisy (*Bellis perennis*), garlic (*Allium sativum*), 'lagaña de perro' (*Caesalpinia gilliesii*), periwinkle (*Catharanthus roseus*), 'rama negra' (*Conyza bonariensis*), 'romerillo' (*Heterothalamus alienus*), summer squash (*Cucurbita maxima* var. zapallito) and tomato (*Solanum lycopersicum*), are new phytoplasma strains while two strains, detected in garlic and China tree (*Melia azedarach*), have been previously described. The plants showed typical symptoms of phytoplasma diseases, such as leaf size reduction, proliferation, stunting and virescence. The identification and genetic diversity analysis of the phytoplasmas were performed based on 16S rDNA and ribosomal protein gene sequences. The classification into 16Sr groups and subgroups was established by actual and virtual RFLP analysis of the PCR products (R16F2/R16R2) compared with reference strains. According to the classification scheme, strains HetLL and ConWB-A and B represent two new subgroups 16SrIII-W and X, respectively. On the other hand, strains CatLL, TomLL and CaesLL are related to subgroup 16SrIII-B, and strains BellVir, TomRed, CucVir and GDIII-207 are related to subgroup 16SrIII-J. Ribosomal protein genes were amplified using primers rpF1/rpR1 and rpIIIF1/rpIIIR1. RFLP analysis performed with *Alu*I, *Dra*I and *Tru1*I (*Mse*I isoschizomer) distinguished three new rp profiles within subgroup 16SrIII-B, one for subgroup 16SrIII-J, and one shared with strains of the new subgroups 16SrIII-W and X. The phylogenetic analysis based on 16S rDNA and ribosomal protein gene sequences confirmed the separation of HetLL and ConWB strains in two new subgroups and the close relatedness among subgroup J phytoplasmas, which have been detected only in South America.

Keywords X-disease · 16S rDNA · Ribosomal proteins · '*Ca*. Phytoplasma pruni'

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Introduction

Phytoplasmas are cell wall-less prokaryotes that have been associated with plant diseases. A wide diversity of phytoplasmas has been detected and classified by sequence analysis of evolutionarily conserved genes. Based on 16S rDNA RFLP analysis with 17 restriction enzymes, Lee et al. (1993, 1998) constructed a comprehensive classification scheme for phytoplasmas. Over thirty 16Sr groups have been described based on actual or in silico RFLP analysis of PCR-amplified 16S rDNA (Zhao et al. 2010). In 2004 the International Research Programme on Comparative Mycoplasmology (IRPCM)

proposed the provisional genus level taxon '*Candidatus* Phytoplasma' to accommodate plant pathogenic, non-helical mollicutes (IRPCM 2004). Over 30 '*Ca*. Phytoplasma' species have been described since then. The most numerous groups are the 16SrI, or Aster yellows, corresponding to '*Ca*. Phytoplasma asteris' and the 16SrIII or X-disease group, for which '*Ca* Phytoplasma pruni' has been provisionally assigned (Davis et al. 2013). An updated classification of 16SrIII group based on in silico RFLP of rDNA sequences described 18 16SrIII subgroups (Zhao et al. 2009). New subgroups 16SrIII-U and V have been later described in Brazil, represented by phytoplasma strain EB02-Br06 that causes eggplant giant calyx disease and PassWB-Br4 that causes passion fruit witches' broom, respectively (Amaral Mello et al. 2011; Davis et al. 2012). Additional phylogenetic markers such as ribosomal protein (rp), *secY* and *tuf* genes have been successfully used for finer differentiation of phytoplasmas. In particular, rp genes have supported the high diversity of group 16SrIII (Gundersen et al. 1996; Martini et al. 2007; Davis et al. 2013).

The first phytoplasma reported in Argentina was the China tree decline phytoplasma (ChTDIII strain), a 16SrIII-group phytoplasma associated with decline and yellowing of *Melia azedarach* (Vázquez et al. 1983; Gomez et al. 1996; Galdeano et al. 2004). Since then, the disease has not only produced an important decrease in *M. azedarach* forest production and implantation but also caused the death of many ornamental trees from city streets and parks around the country. Phytoplasma decline and yellowing disease of *M. azedarach* has been reported in other South American countries such as Bolivia (Harrison et al. 2003), Paraguay (Arneodo et al. 2007) and Brazil (Duarte et al. 2009). Another 16SrIII-group phytoplasma (Garlic decline phytoplasma, GDIII strain) has been detected in garlic causing a disease named 'tristeza del ajo', which causes leaf reddening or yellowing depending on the cultivar, wilt and plant death (Galdeano et al. 2004). The disease has been repeatedly detected with low incidence in all the garlic producing areas of Argentina (Galdeano et al. 2009). After that, 16SrIII-group phytoplasmas were detected in different cultivated plant species such as the recently published Argentinean peach yellows (Fernández et al. 2013), and other not-yet described from tomato, summer squash, periwinkle and daisy. Up to now the occurrence of these diseases has not been regular along the years and restricted to some particular locations. Likewise, phytoplasmas of the same group have been detected in three naturally growing plant species, *Caesalpinia gilliesi*, *Heterothalamus alienus* and *Conyza bonariensis*.

The aim of this work was to identify ten new phytoplasmas detected in Argentina, analyze the diversity of the 16SrIII-group phytoplasmas from Argentina, and establish the phylogenetic relationships among them and other 16SrIII group phytoplasma strains.

Materials and methods

Plant samples and reference phytoplasma strains

Leaf and petiole samples were collected from symptomatic and asymptomatic China tree (*Melia azedarach*), daisy (*Bellis perennis*), garlic (*Allium sativum*), 'lagaña de perro' (*Caesalpinia gilliesii*), periwinkle (*Catharanthus roseus*), 'rama negra' (*Conyza bonariensis*), 'romerillo' (*Heterothalamus alienus*), summer squash (*Cucurbita maxima* var. zapallito) and tomato (*Solanum lycopersicum*) in different localities of Argentina (Table 1).

Genomic DNA was purified from symptomatic and asymptomatic leaves and petioles by the CTAB technique (Doyle and Doyle 1990) and used as a template in polymerase chain reactions (PCRs) for amplification of 16S ribosomal RNA and ribosomal protein gene sequences.

PCR and RFLP analysis

Ribosomal DNA from the phytoplasma strains was amplified by direct PCR using universal primers P1/P7 (Deng and Hiruki 1991; Schneider et al. 1995) or R16F2/R16R2 (Lee et al. 1993) under the conditions previously described (Galdeano et al. 2004). Western X (WX) phytoplasma (provided by Dr. E. Seemüller) was used as 16SrIII group reference strain. For RFLP analysis, R16F2/R16R2 PCR products (1.24 kb fragments) were separately digested with enzymes *Alu*I (Promega), *BstU*I, *Hae*III, *Hha*I, *Rsa*I, *Taq*I (New England Biolabs) and *Tru1*I (*Mse*I isoschizomer) (Thermo Scientific) according to the manufacturer's instructions. China-tree decline (ChTDIII) and Garlic decline (GDIII) phytoplasmas, which have been previously reported as 16SrIII-B and J subgroup related strains, respectively (Galdeano et al. 2004), were also included for comparison. Ribosomal protein genes including 3′ end of *rps19*, *rplV* (*rpl22*), *rpsC* (*rps3*) genes and 5′

Table 1 Classification, host and geographic location of the 16SrIII-group phytoplasma strains used in the present paper, based on RFLP analysis of 16S rDNA

Phytoplasma strain	Plant host	Geographical origin[a]	16SrIII subgroup	16S rDNA Highest similarity coefficient (subgroup)[b]
Western X (WX)	*Prunus persica*	USA	S[c]	
Garlic decline (GDIII)	*Allium sativum*	San Juan, Argentina (CW)	J[d]	1 (J)
Garlic decline (GDIII-207)	*Allium sativum*	San Juan, Argentina (CW)	J	1 (J)
Tomato reddening (TomRed)	*Solanum lycopersicum*	San Juan, Argentina (CW)	J	1 (J)
Bellis virescence (BellVir)	*Bellis perennis*	Córdoba, Argentina (C)	J	1 (J)
Cucurbita virescence (CucVir)	*Cucurbita maxima* var. zapallito	Córdoba, Argentina (C)	J	0.97 (J)
China-tree decline (ChTDIII)	*Melia azedarach*	Corrientes, Argentina (NE)	B[d]	1 (B)
Tomato little leaf (TomLL)	*Solanum lycopersicum*	Tucumán, Argentina (NW)	B	1 (B)
Caesalpinia little leaf (CaesLL)	*Caesalpinia gilliesii*	Córdoba, Argentina (C)	B	0.97 (B)
Catharanthus little leaf (CatLL)	*Catharanthus roseus*	Corrientes, Argentina (NE)	B	1 (B)
Conyza witches' broom strain A (ConWB-A)	*Conyza bonariensis*	Córdoba, Argentina (C)	X	0.97 (B)
Conyza witches' broom strain B (ConWB-B)	*Conyza bonariensis*	Córdoba, Argentina (C)	X	0.97 (B)
Heterothalamus little leaf (HetLL)	*Heterothalamus alienus*	Córdoba, Argentina (C)	W	0.94 (B)

[a] *NE* North East; *CW* Central West; *NW* North West; *C* Central Argentina

[b] Results retrieved from the *i*PhyClassifier (Zhao et al. 2009)

[c] Zhao et al. 2009

[d] Galdeano et al. 2004

end of *rplP* (*rpl16*) gene were amplified using primers rpF1/rpR1 (Lim and Sears 1992; Gundersen et al. 1996) and X-disease group specific primers rpIIIF1/rpIIIR1 (Martini et al. 2007). PCR fragments amplified with the latter primers were digested with *Alu*I, *Dra*I (New England Biolabs) and *Tru1*I (*Mse*I isoschizomer) (Thermo Scientific) endonucleases. Restriction fragments were resolved in 10 % PAGE (12 % for *Tru1*I digested rp fragment) buffered in Tris-borate EDTA, stained with ethidium bromide and observed under ultraviolet light.

Nucleotide sequencing, putative restriction site and phylogenetic analysis

16S rDNA and rp genes of phytoplasma strains GDIII-207 (Garlic decline), TomLL (Tomato little leaf), TomRed (Tomato red leaf), CaesLL (Caesalpinia little leaf), BellVir (Bellis virescence), CatLL (Catharanthus little leaf), ConWB-A, ConWB-B (Conyza witches' broom), HetLL (Heterothalamus little leaf), CucVir (Cucurbita virescence), amplified using GoTaq DNA polymerase (Promega), were cloned and sequenced. As regards ribosomal genes, ChTDIII and GDIII strains were also included.

Amplified fragments of 16S rDNA and ribosomal protein genes were cloned using pGEM®-T Easy Vector System II (Promega). The clones obtained were sequenced with an automatic sequencer and the sequences were assembled using SeqMan program (Lasergene, DNASTAR) and uploaded to the NCBI GenBank (http://www.ncbi.nlm.nih.gov).

The phytoplasma classification into 16Sr groups and subgroups was established by the putative RFLP similarity coefficient analysis and virtual gels were constructed using the *i*PhyClassifier on line program (Zhao et al. 2009) The 16S rDNA sequences were trimmed to an approximately 1.24 kb fragment, comprised between R16F2 and R16R2 primers (Lee et al. 1998). In silico RFLP patterns resulting from digestions with enzymes *Alu*I, *Dra*I and *Mse*I were performed for the ribosomal protein gene sequences of the 16SrIII Argentinean phytoplasmas using the pDRAW32 program developed by Aca Clone Software (www.acaclone.com). Sequence similarity was calculated after Clustal W multiple sequence alignment using MegAlign

program (DNASTAR, Lasergene). Phylogenetic and molecular evolutionary analyses were conducted using MEGA version 5 (Tamura et al. 2011).

16S rDNA and protein gene sequences of the Argentinean 16SrIII-group phytoplasma strains, including the ones generated in this work and the previously reported ChTDIII and GDIII were aligned with those of other 16SrIII-group phytoplasmas obtained from the GenBank database. Phylograms were generated to estimate the phylogenetic relationships based on the neighbour-joining method, with the reliability provided by 1,000 replications bootstrap test.

Results

Plant symptoms and phytoplasma detection

Most of the infected hosts showed typical phytoplasma symptoms such as leaf size reduction and internode shortening. The tomatoes infected with TomRed strain showed leaf reddening with coriaceous appearance. The most visible symptom of infected summer squash and daisies was virescence while flower bud proliferation was observed only in *C. bonariensis* infected with ConWB phytoplasma strains.

Phytoplasma RFLP identification

Phytoplasmas were detected in all DNA samples extracted from symptomatic plants by direct PCR using universal primers P1/P7, R16F2/R16R2 for rDNA and the 16SrIII specific primers rpIIIF1/rpIIIR1 for ribosomal protein genes. No positive reactions were obtained from asymptomatic samples.

All the assayed phytoplasma strains had identical 16Sr DNA RFLP patterns compared to the WX reference strain for *Alu*I, *BstU*I (data not shown) and *Tru1*I enzymes (Fig. 1). ChTDIII (16SrIII-subgroup B), TomLL, CaesLL, CatLL, ConWB-A, ConWB-B and HetLL strains had *Hha*I patterns identical to the WX phytoplasma and different to GDIII (16SrIII-subgroup J), GDIII-207, TomRed, BellVir and CucVir strains (Fig. 1). Strain CucVir showed a particular restriction pattern for *Taq*I (Fig. 1), different from all the phytoplasmas assayed. Strains ConWB-A, ConWB-B and HetLL had the same *Rsa*I pattern which was different from all the phytoplasmas assayed, and phytoplasma HetLL had also a unique *Hae*III pattern (Fig. 2).

Restriction analysis of ribosomal protein gene fragments amplified with rpIIIF1/rpIIIR1 primers was performed with enzymes *Dra*I, *Alu*I and *Tru1*I (Fig. 3). All the Argentinean phytoplasmas had the similar *Dra*I pattern, different from that of the WX pyhtoplasma; however, *Alu*I digestions resulted in four different profiles. The most numerous group was composed by phytoplasmas GDIII, GDIII-207, TomRed, BellVir, CucVir and TomLL which had the same profile. ChTDIII phytoplasma showed a pattern almost identical to the later, but with the highest band approximately 20 bp smaller. Strains CaesLL and CatLL shared a common *Alu*I profile different from the other strains. The last profile group was composed of strains ConWB-A and B, and HetLL, which were similar to the reference strain WX. As regards *Tru1*I restriction enzyme, most of the assayed strains had a pattern indistinguishable from the WX reference strain. Two other different patterns were observed for this enzyme, one for ConWB-A and B, and HetLL strains while the other one was unique for TomLL strain.

Sequence similarity and putative restriction sites in 16S rDNA and ribosomal protein genes

The nucleotide sequences of 16S rDNA and ribosomal protein genes from the phytoplasma strains analyzed in this work were deposited in the GenBank database. The rDNA sequence homology analysis distinguished two groups among the Argentinean 16SrIII phytoplasmas. TomRed, TomLL, BellVir, CatLL, CaesLL and CucVir phytoplasmas showed high sequence homology among them and with phytoplasmas of subgroups J (GDIII) and B (ChTDIII), with maximum between 99.8 and 99.4 and minimum between 99.4 and 99.1. On the other hand, phytoplasmas HetLL, ConWB-A and ConWB-B showed the highest sequence similarity between them (99.6) followed by CYE phytoplasma (99.3 and 99.4, respectively).

In silico restriction site maps were coherent with the real PCR-RFLP profiles obtained and confirmed the differential patterns of phytoplasmas CucVir, HetLL and ConWB (Figs. 4 and 5). The differential *Taq*I pattern presented by phytoplasma CucVir corresponded with a C insertion in position 994. In the case of *Rsa*I patterns of HetLL and ConWB, and *Hae*III pattern of HetLL, they reflected base substitutions in the nucleotide sequences (data not shown).

According to the similarity coefficient comparison performed using the *i*PhyClassifier, ChTDIII, TomLL,

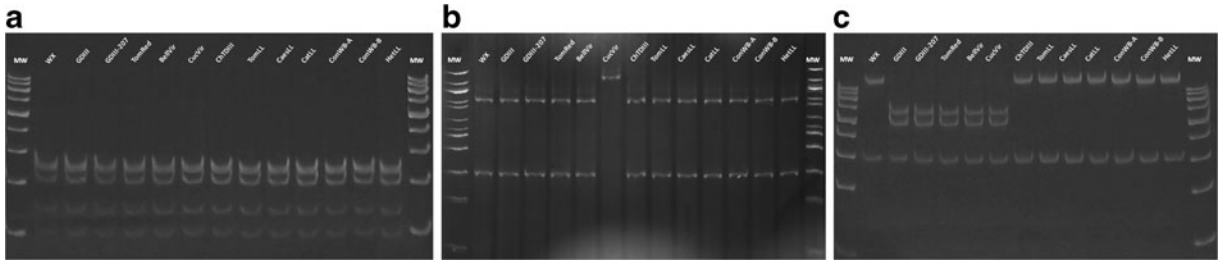


Fig. 1 RFLP analysis of R16F2/R16R2 PCR products of 16S rRNA gene digested with *Tru1*I (**a**), *Taq*I (**b**) and *Hha*I (**c**) restriction enzymes. Phytoplasma strains and 16SrIII subgroup: WX, Western-X (16SrIII-S); GDIII Garlic decline (16Sr III-J); GDIII-207, Garlic decline strain 207 (16Sr III-J); TomRed, Tomato red leaf (16SrIII-J); BellVir, Bellis virescence (16SrIII-J); CucVir, Cucurbita virescence (16SrIII-J); ChTDIII, China tree decline (16Sr III-B); TomLL, Tomato little leaf (16SrIII-B); CaesLL, Caesalpinia little leaf (16SrIII-B); CatLL, Catharanthus little leaf (16SrIII-B); ConWB-A and ConWB-B, Conyza witches' broom, isolates A and B (16SrIII-X); HetLL, Heterothalamus little leaf (16SrIII-W). Molecular Weight Marker A and C: Gene Ruler 100 bp DNA ladder (Thermo Scientific) 100, 200, 300, 400, 500, 600, 700, 800, 900, 1,000 bp. B: 100 bp DNA ladder (New England Biolabs) 100, 200, 300, 400, 500/517, 600, 700, 800, 900, 1,000, 1,200, 1,517 bp

CatLL and CaesLL phytoplasmas are strains related with subgroup 16SrIII-B while GDIII-207, TomRed and BellVir are related to subgroup 16SrIII-J. Phytoplasmas CucVir, HetLL and ConWB are related to the 16SrIII group, but are different from all the known subgroups (Table 1).

Ribosomal protein sequence analysis showed that phytoplasmas from subgroup J had higher than 99 % sequence homology among them while phytoplasmas from subgroup B had sequence homology of 98–99 % among them and with subgroup J phytoplasmas. Strains ConWB and HetLL had 98.8 % homology between them, higher than 97 % with WX and SP1 (Spirea stunt) phytoplasmas, and lower than 97 % with the other Argentinean phytoplasmas.

In silico restriction site maps were coherent with the actual PCR-RFLP profiles obtained with enzymes *Alu*I, *Dra*I and *Tru1*I (*Mse*I) (Fig. 6). All the phytoplasmas of subgroup 16SrIII-J had the same rp patterns for each of the three enzymes assayed. Subgroup 16SrIII-B phytoplasmas were more variable: CaesLL and CatLL strains had identical patterns; ChTDIII strain shared *Dra*I and *Tru1*I patterns with them, but had a differential *Alu*I pattern. Phytoplasma TomLL had the same patterns as subgroup 16SrIII-J phytoplasmas with enzymes *Dra*I and *Alu*I, but a different pattern with *Tru1*I. Phytoplasmas HetLL and ConWB-A and B had a profile consisting of the *Alu*I pattern similar to WX reference strain, and the same *Dra*I pattern as the Argentinean strains assayed.

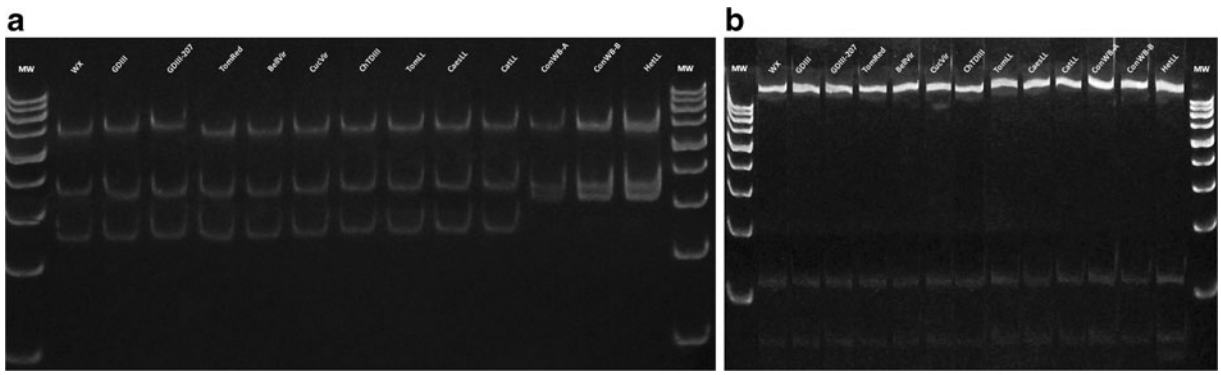


Fig. 2 RFLP analysis of R16F2/R16R2 PCR products of 16SrRNA gene digested with *Rsa*I (**a**) and *Hae*III (**b**) restriction enzymes. Phytoplasma strains and 16SrIII subgroup: WX, Western-X (16SrIII-S); GDIII Garlic decline (16Sr III-J); GDIII-207, Garlic decline strain 207 (16Sr III-J); TomRed, Tomato red leaf (16SrIII-J); BellVir, Bellis virescence (16SrIII-J); CucVir, Cucurbita virescence (16SrIII-J); ChTDIII, China tree decline (16Sr III-B); TomLL, Tomato little leaf (16SrIII-B); CaesLL, Caesalpinia little leaf (16SrIII-B); CatLL, Catharanthus little leaf (16SrIII-B); ConWB-A and ConWB-B, Conyza witches' broom, isolates A and B (16SrIII-X); HetLL, Heterothalamus little leaf (16SrIII-W). MW: Gene Ruler 100 bp DNA ladder (Thermo Scientific) 100, 200, 300, 400, 500, 600, 700, 800, 900, 1,000 bp

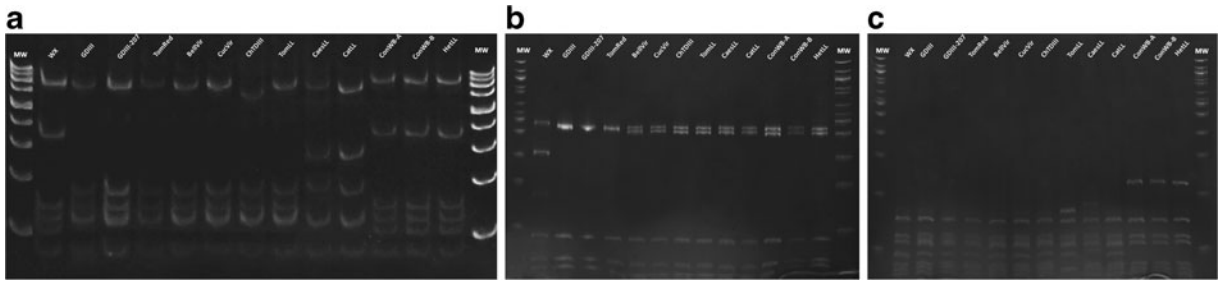


Fig. 3 RFLP patterns of ribosomal protein genes rpIIIF/rpIIIR fragments digested with *Alu*I (**a**), *Dra*I (**b**) and *Tru1*I (**c**) restriction enzymes. Phytoplasma strains WX (Western-X); GDIII (Garlic decline); GDIII-207 (Garlic decline strain 207); TomRed (Tomato red leaf); BellVir (Bellis virescence); CucVir (Cucurbita virescence); ChTDIII (China tree decline); TomLL (Tomato little leaf); CaesLL (Caesalpinia little leaf); CatLL (Catharanthus little leaf); ConWB-A and ConWB-B (Conyza witches' broom, isolates A and B); HetLL (Heterothalamus little leaf). Molecular Weight Marker A: Gene Ruler 100 bp DNA ladder (Thermo Scientific) 100, 200, 300, 400, 500, 600, 700, 800, 900, 1,000 bp. B and C: 100 bp DNA ladder (New England Biolabs) 100, 200, 300, 400, 500/517, 600, 700, 800, 900, 1,000, 1,200, 1,517 bp

Phylogenetic analysis

The phylogenetic tree constructed by neighbour-joining analysis based on 16S rDNA sequences of 31 16SrIII-group phytoplasma strains, 12 Argentinean isolates, six phytoplasmas representative of different 16Sr groups, and *Acholeplasma modicum* as outgroup confirmed that the phytoplasma strains included in this study are related to group 16SrIII. The phytoplasma strains from subgroups 16SrIII-J (GDIII, GDIII-207, TomRed, CucVir, BellVir) and B (ChTDIII, TomLL, CatLL and CaesLL) clustered together and with strains representative of subgroups 16SrIII-U, R and F. HetLL and ConWB strains, corresponding to new 16SrIII subgroups W and X, conformed instead, a clearly separated branch (Fig. 7).

For the phylogenetic analysis of ribosomal protein gene sequences, a neighbour-joining tree was constructed with 12 Argentinean strains, 12 sequences of 16SrIII-

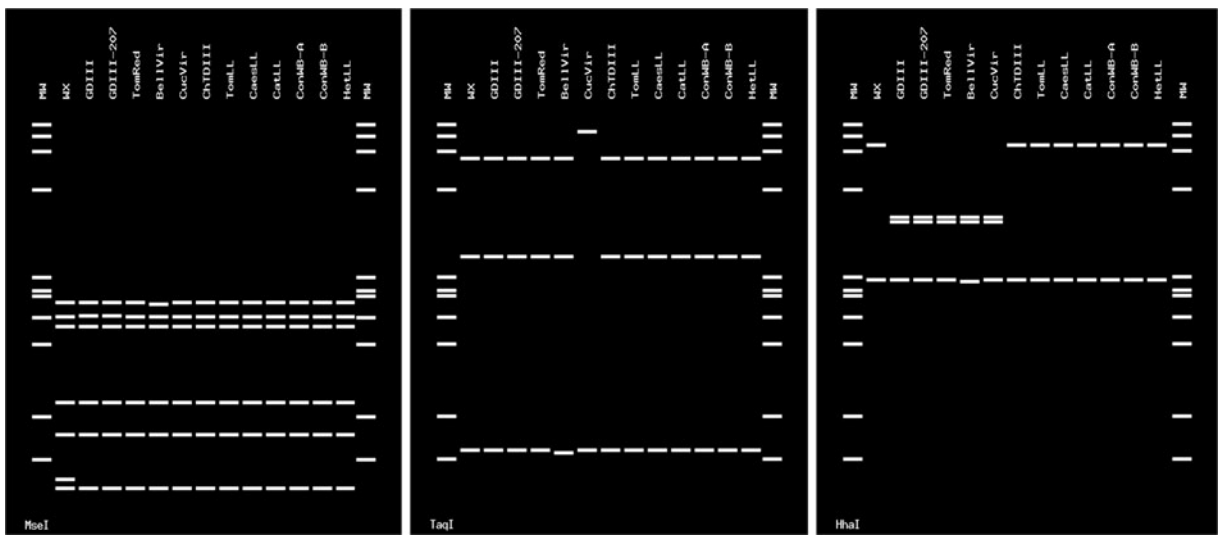


Fig. 4 Virtual RFLP patterns of partial 16SrRNA gene constructed using the *i*PhyClassifier on line program (Zhao et al. 2009). Recognition sites for restriction enzymes *Mse*I, *Taq*I and *Hha*I. Phytoplasma strains and GenBank accession number. WX, Western-X (L04682); GDIII Garlic decline (AY081816); GDIII-207, Garlic decline strain 207 (KC412032); TomRed, Tomato red leaf (KC412031); BellVir, Bellis virescence (KC412024); CucVir, Cucurbita virescence (KC412028); ChTDIII, China tree decline (AY081817); TomLL, Tomato little leaf (KC412025); CaesLL, Caesalpinia little leaf (KC202812); CatLL, Catharanthus little leaf (KC412025); ConWB-A and ConWB-B, Conyza witches' broom, isolates A and B (KC412026; KC412027); HetLL, Heterothalamus little leaf (KC412029). MW: øX174 DNA-HaeIII digested

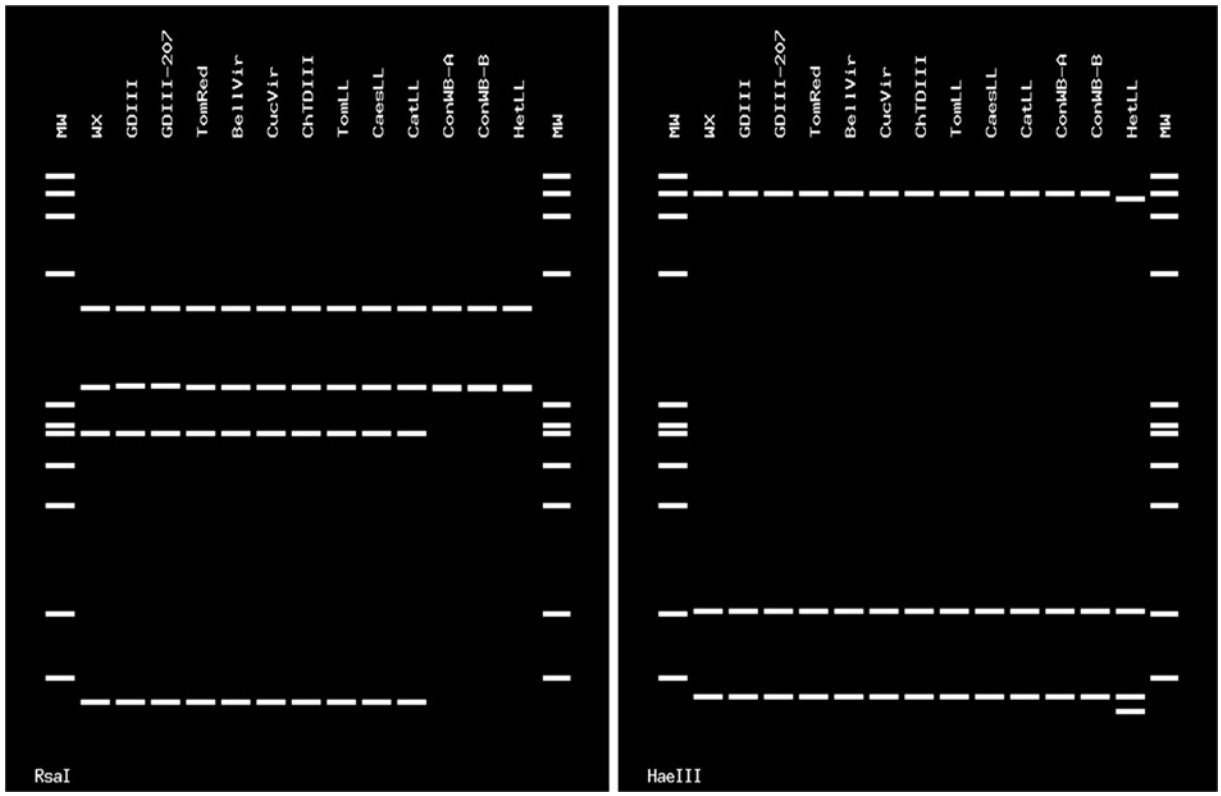


Fig. 5 Virtual RFLP patterns of partial 16SrRNA gene constructed using the *i*PhyClassifier on line program (Zhao et al. 2009). Recognition sites for restriction enzymes *Hae*III and *Rsa*I. Phytoplasma strains and GenBank accession number. WX, Western-X (L04682); GDIII Garlic decline (AY081816); GDIII-207, Garlic decline strain 207 (KC412032); TomRed, Tomato red leaf (KC412031); BellVir, Bellis virescence (KC412024); CucVir, Cucurbita virescence (KC412028); ChTDIII, China tree decline (AY081817); TomLL, Tomato little leaf (KC412025); CaesLL, Caesalpinia little leaf (KC202812); CatLL, Catharanthus little leaf (KC412025); ConWB-A and ConWB-B, Conyza witches' broom, isolates A and B (KC412026; KC412027); HetLL, Heterothalamus little leaf (KC412029). MW: øX174 DNA-HaeIII digested

group phytoplasmas, 13 from other groups and *A. palmae* as outgroup. The tree showed that 16SrIII group phytoplasmas conformed a cluster well separated from other groups. Within the 16SrIII group, two main clusters could be distinguished. One of them included the ribosomal protein sequences of subgroups 16SrIII-J and B, and the other grouped the remaining 16SrIII subgroups, including those of phytoplasmas HetLL and ConWB (new subgroups 16SrIII-W and X) (Fig. 8).

Discussion

The X-disease (16SrIII) is one of the most numerous phytoplasma groups; not only does it have a high amount of strains and subgroups but also a wide host range. Among the 21 16SrIII subgroups described (Zhao et al. 2009; Amaral Mello et al. 2011), only subgroups J and B have been reported up to now in Argentina (Galdeano et al. 2004; Fernández et al. 2013). In this work we present the molecular characterization of 9 new 16SrIII phytoplasma strains based on 16S rDNA and ribosomal protein gene analysis. The results confirm that subgroups 16SrIII-J and B are the most common in Argentina, similar to the situation reported in Brazil (Amaral Mello et al. 2011; Montano et al. 2011). In particular, subgroup 16SrIII-J has been detected until now only in South America infecting many plant species, such as *Sechium edule*, *Momordica charantia*, *Luffa cylindrica*, *Sicana odorifera*, *Cucurbita moschata*, *Allium sativum*, *Manihot esculenta*, *Prunus avium* and *Solanum melongena* (Montano et al. 2000; Alvarez et al. 2004; Galdeano et al. 2004; Amaral Mello et al. 2011; González et al. 2011). In this work we report new hosts for subgroup

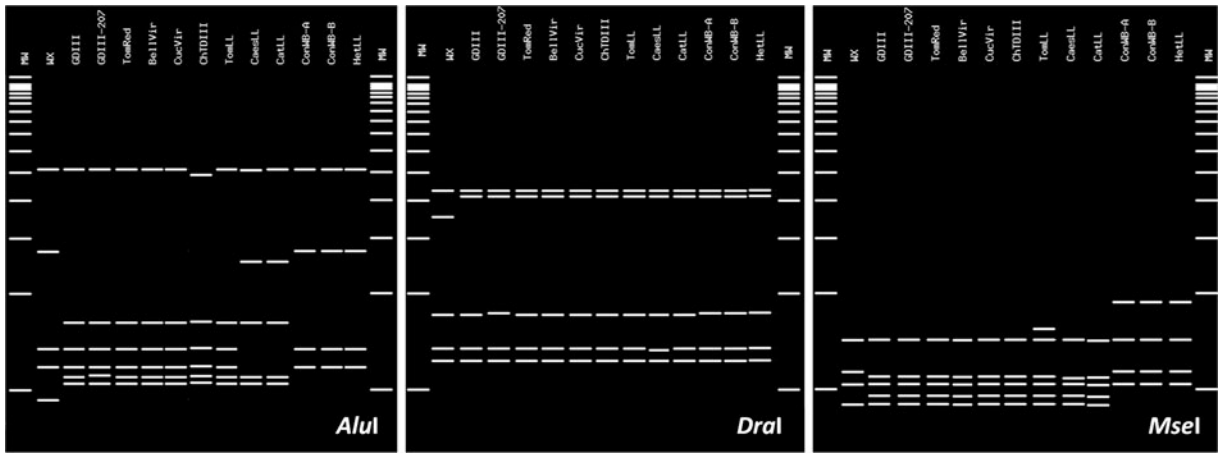
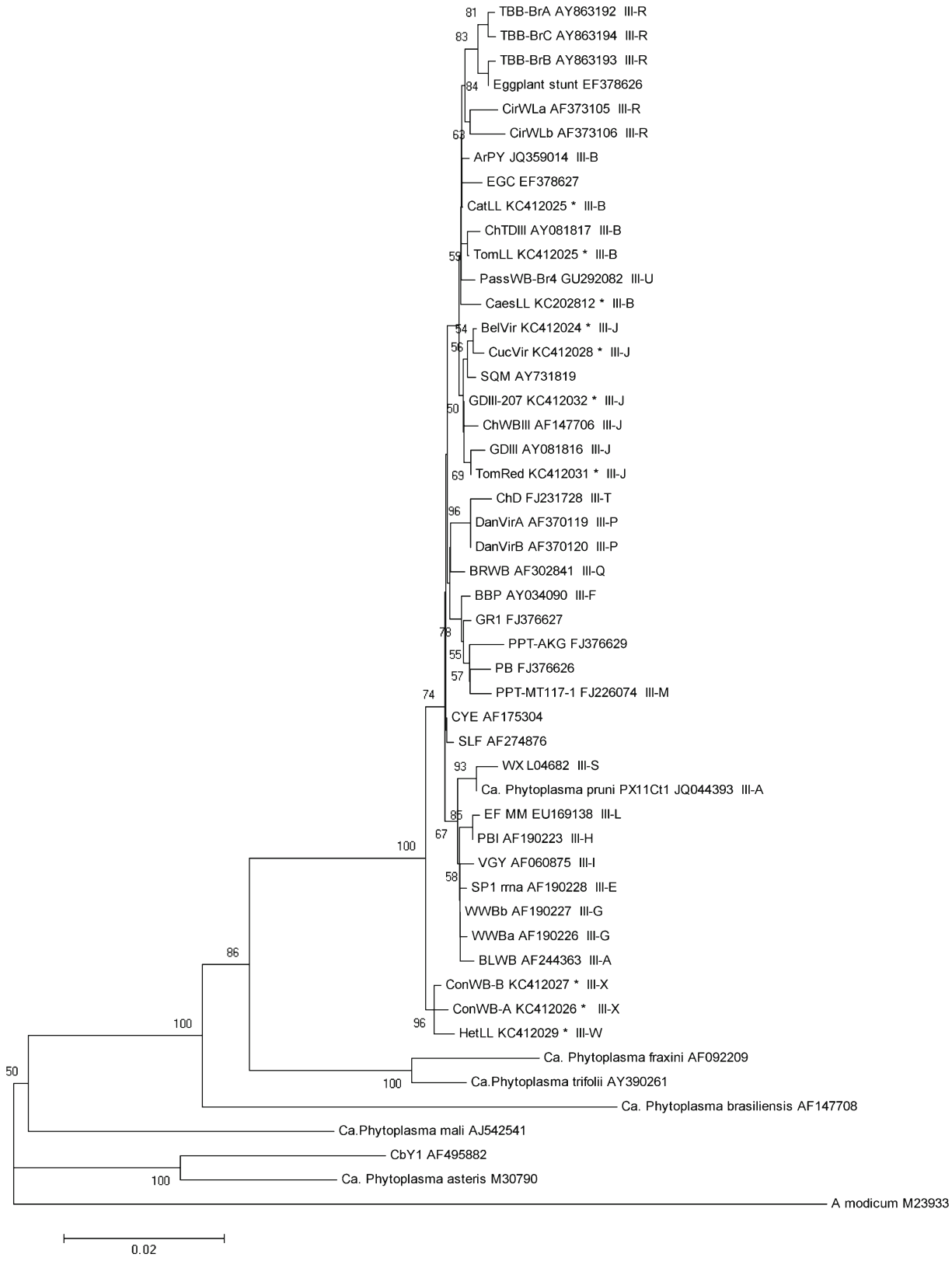


Fig. 6 Virtual RFLP patterns of ribosomal protein genes rpIIIF/rpIIIR fragments of 16SrIII-group phytoplasmas. Recognition sites for restriction enzymes *Alu*I, *Dra*I and *Mse*I were used in the simulated digestions. Phytoplasma strains and GenBank accession numbers: WX (Western X- JQ360956); GDIII (Garlic decline- KC412014); GDIII-207 (KC417336); TomLL (Tomato little leaf- KC412018); BellVir (Bellis virescence- KC412019); CucVir (Cucumber virescence- KC412017); ChTDIII (China tree decline- KC412013); TomRed (Tomato reddening- KC412023); CaesLL (Caesalpinia little leaf- KC412021); CatLL (Catharanthus little leaf- KC412022); ConWB-A (Conyza witches' broom—KC412015); ConWB-B (Conyza witches' broom—KC412016); HetLL (Heterothalamus little leaf—KC412020). MW: 100 bp DNA ladder (Invitrogen) 100, 200, 300, 400, 500, 600, 700, 800, 900, 1,000, 1,100, 1,200, 1,300, 1,400, 1,500, 2,072 bp

16SrIII-J, *Bellis perennis*, and subgroup 16SrIII-B, *Caesalpinia gilliesii*. A phytoplasma disease of *Bellis perennis*, caused by an aster yellows-group phytoplasma has been previously reported in Lithuania showing similar symptoms to those described in this paper (Samuitiene et al. 2002). Our results indicate that *B. perennis* can also host a phytoplasma from the 16SrIII-J subgroup, increasing the wide host range of this subgroup. The results showed that subgroup 16SrIII-J is very homogeneous considering 16S rDNA and ribosomal protein genes since all the related strains have equal RFLP patterns and high sequence homology.

According to the 16S rDNA RFLP analysis, if a phytoplasma strain has 0.97 or lower similarity coefficient with those of all strains representative of a certain group, it could be assigned to a new subgroup (Wei et al. 2008). Three of the phytoplasmas described in this work, CucVir, HetLL and ConWB, have similarity coefficients equal to or lower than 0.97 with other 16SrIII group members. Phytoplasmas HetLL and ConWB represent new 16SrIII subgroups W and X, respectively. Both phytoplasmas share a differential *Rsa*I pattern. HetLL has also a particular *Hae*III pattern. Besides, the phylogenetic tree based on rDNA sequences clustered phytoplasmas HetLL and ConWB in a separate branch within 16SrIII phytoplasmas. No previous reports exist for phytoplasma infected *Heterothalamus alienus* (Spreng.) Kuntze (Asteraceae). This is a perennial shrub endemic of Central Argentina, Southern Brazil and Uruguay. Although not commercially produced, *H. alienus* is a medicinal species commonly used for treating fever, kidney diseases and as a stimulant; also anti fungal activity has been reported in leaf extracts (Carpinella et al. 2010). Unlike the previous species, *Conyza bonariensis* (L.) Cronquist (synonym: *Erigeron bonariensis*) (Asteraceae) has been reported to host phytoplasmas related to group 16SrVII in Brazil and Argentina (Barros et al. 2002; Meneguzzi et al. 2008). Our results show that *C. bonariensis* is also host of a new phytoplasma of group 16SrIII new subgroup III-X. In fields from Córdoba province (Central Argentina), where ConWB phytoplasma has been detected in many plants growing as weeds in peach orchards and HetLL in plants naturally growing in nearby areas, the phytoplasmas have not been detected in peach trees or other crops up to the moment. However, in fields from Jujuy province (North Western Argentina), a phytoplasma characterized as a subgroup 16SrIII-B member has been detected in peach

Fig. 7 Phylogenetic tree constructed by neighbour-joining analysis based on 16S rDNA gene sequences including 31 phytoplasma strains representative of 16SrIII subgroups, the 12 Argentinean isolates and six phytoplasmas representative of different 16Sr groups. *A. modicum* was used as outgroup. Bootstrap values are shown on *branches*. *Asterisks* (*) indicate new sequences analyzed in this work. *Capital letters on the right* indicate 16SrIII subgroups ▶


TBB-BrA AY863192 III-R
TBB-BrC AY863194 III-R
TBB-BrB AY863193 III-R
Eggplant stunt EF378626
CirWLa AF373105 III-R
CirWLb AF373106 III-R
ArPY JQ359014 III-B
EGC EF378627
CatLL KC412025 * III-B
ChTDIII AY081817 III-B
TomLL KC412025 * III-B
PassWB-Br4 GU292082 III-U
CaesLL KC202812 * III-B
BelVir KC412024 * III-J
CucVir KC412028 * III-J
SQM AY731819
GDIII-207 KC412032 * III-J
ChWBIII AF147706 III-J
GDIII AY081816 III-J
TomRed KC412031 * III-J
ChD FJ231728 III-T
DanVirA AF370119 III-P
DanVirB AF370120 III-P
BRWB AF302841 III-Q
BBP AY034090 III-F
GR1 FJ376627
PPT-AKG FJ376629
PB FJ376626
PPT-MT117-1 FJ226074 III-M
CYE AF175304
SLF AF274876
WX L04682 III-S
Ca. Phytoplasma pruni PX11Ct1 JQ044393 III-A
EF MM EU169138 III-L
PBI AF190223 III-H
VGY AF060875 III-I
SP1 rrna AF190228 III-E
WWBb AF190227 III-G
WWBa AF190226 III-G
BLWB AF244363 III-A
ConWB-B KC412027 * III-X
ConWB-A KC412026 * III-X
HetLL KC412029 * III-W
Ca. Phytoplasma fraxini AF092209
Ca.Phytoplasma trifolii AY390261
Ca. Phytoplasma brasiliensis AF147708
Ca.Phytoplasma mali AJ542541
CbY1 AF495882
Ca. Phytoplasma asteris M30790
A modicum M23933
0.02

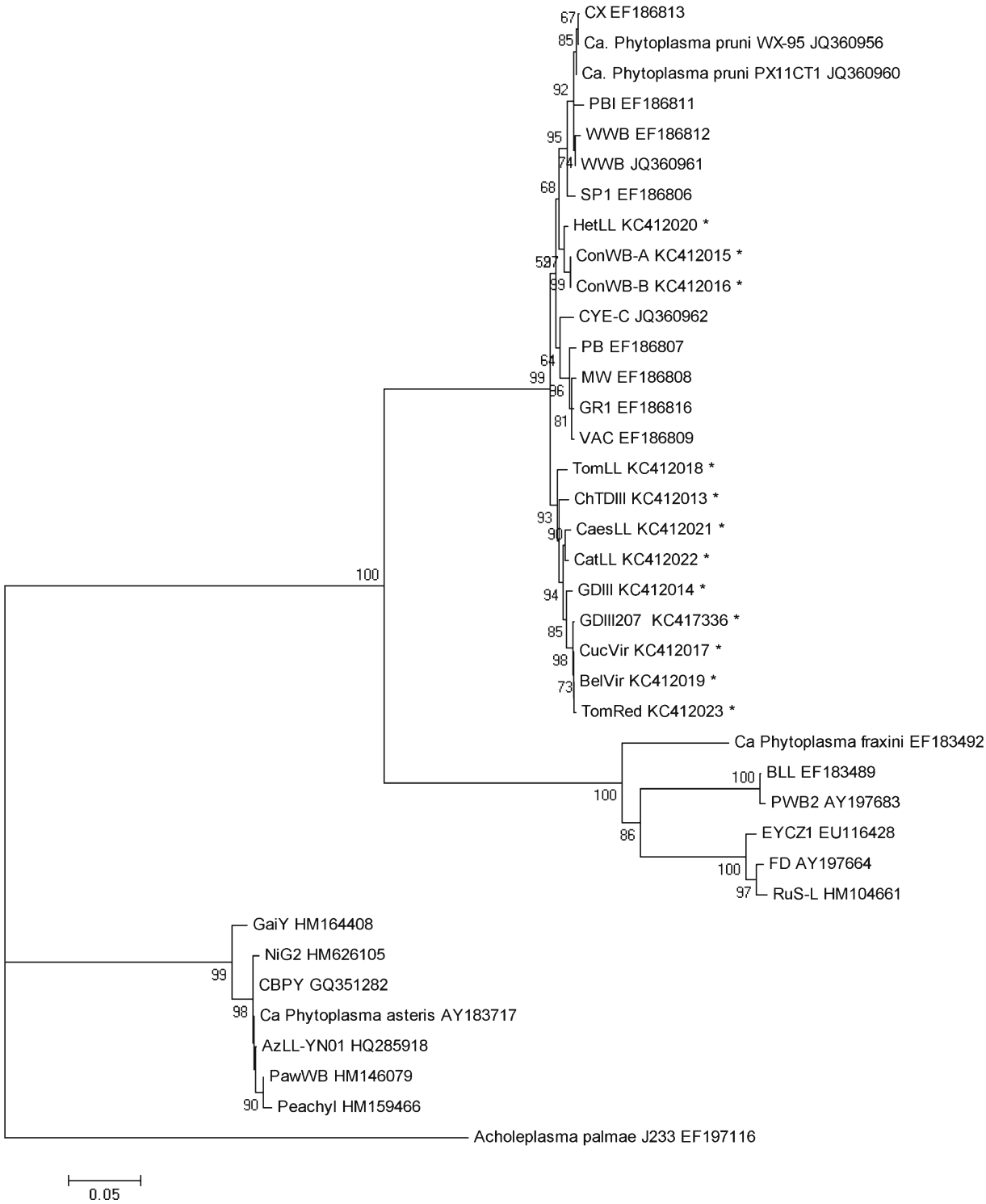


Fig. 8 Phylogenetic tree constructed by neighbour-joining analysis based on ribosomal protein (rp) gene sequences including 12 sequences of 16SrIII-group representative phytoplasmas, the 12 Argentinean isolates analyzed in this paper and 13 phytoplasmas representative of different 16Sr groups. *A. modicum* was used as outgroup. Bootstrap values are shown on *branches*. *Asterisks* (*) indicate new sequences analyzed in this work

trees inducing important losses (Fernández et al. 2013). We do not know whether this responds to pathogen specificity or to the lack of a suitable vector, but the regular detection of phytoplasmas in plants growing wild represents a potential inoculum source for cultivated species.

CucVir phytoplasma can be distinguished by its *Taq*I RFLP pattern which consists of 2 DNA fragments corresponding to 1,100 and 100 bp instead of the 3-band pattern presented by the other phytoplasmas of the 16SrIII group. However, the differences found might not be sufficient to describe a new subgroup, considering as well that the phylogenetic analysis of the rDNA and ribosomal protein gene sequences showed a close relatedness of this phytoplasma with subgroup J strains. Although many cucurbits have been found to host phytoplasmas, this is the first report of a phytoplasma disease in *Cucurbita maxima* var zapallito, a summer squash species native of South America and widely cultivated in central and northern Argentina. Another *Cucurbita* species, *C. moschata* has been reported to host a 16SrIII-J subgroup phytoplasma in Brazil (Montano et al. 2011).

In this work we have also analyzed the sequences of ribosomal protein genes. The RFLP analysis showed that the combined profiles of the 11 strains analyzed were different from previously reported 16SrIII phytoplasmas. In particular, strains from subgroup J had the same patterns for the three restriction enzymes assayed. Another RFLP profile was shared by HetLL and ConWB strains, which correspond to new subgroups W and X. On the other hand, three different profiles were observed among subgroup 16SrIII-B strains. Such diversity has been previously noticed in subgroup B by Gundersen et al. (1996) between strains CYE (Clover yellow edge) and MW (Milkweed yellows). The analysis of the ribosomal protein gene sequences has been useful for discriminating strains within subgroup 16SrIII-B as well as showing the closeness of subgroup 16SrIII-J phytoplasmas. Phylogenetic analyses based on these genes (Martini et al. 2007) and other such as *secY* gene (Lee et al. 2010) have already shown to be useful for further differentiation of phytoplasma strains previously characterized by 16S rDNA.

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