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'*Ca*. Phytoplasma pruni' and '*Ca*. Phytoplasma meliae' are affecting plum in Argentina

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Abstract

We report for the first time the presence of phytoplasmas in association with Plum (*Prunus domestica*) yellowing in Argentina. Molecular analysis of 16S rDNA gene sequence (RFLP, phylogeny) reveals the existence of two different 16Sr-subgroups, 16SrIII-B and novel 16SrXIII-L. These results contribute to a better understanding of the diversity of phytoplasmas associated with *Prunus* genera in South America.

Keywords Plum · Diversity · Detection · 16Sr DNA · Phylogeny · Phytoplasma

Stone fruits are affected by diverse phytoplasmas worldwide (Marcone 2014). Within these, '*Ca*. Phytoplasma prunorum' (16SrX-F) is the major and economically most important phytoplasma affecting plum (*Prunus domestica*) in Europe. This phytoplasma also induces economically significant damages on Japanese plum trees and represents one of the major limiting factors of plum production in Mediterranean countries (Fiore et al. 2018). In temperate areas of South America, different phytoplasmas affecting stone fruit trees have been reported (Quiroga et al. 2018; Fernández et al. 2013, 2017) however; to our knowledge, there has been no reports of infections in plum. During 2019 -2020 typical symptoms of phytoplasma disease, including yellowing, shortening of internodes and decrease in leaves size (Fig. 1a and c) were observed on plum production lots located in Jujuy province (Argentina Northwest region). Also, peach (*Prunus persicae*) plants exhibiting symptoms of phytoplasma infection similar to those previously described by Fernandez et al. (2013), were observed in contiguous plots. Argentina is the third largest producer and exporter of dehydrated plum in the world (Secretaria de agroindustria - Ministerio de Producción y Trabajo, 2019), therefore detecting and identifying the pathogens that affect Plum production is of utmost importance. Hence, the aim of this work is to detect and identify the pathogen associated with the described symptomatology in plum plants and perform its molecular characterization.

Symptomatic plum samples (branches and leaves) ($n = 5$ trees) and symptomatic peach samples ($n = 3$ trees) were collected in contiguous commercial production plots. Asymptomatic tree samples were also collected and used as controls. For DNA isolation of these samples, petioles and midribs were ground with liquid nitrogen according to Doyle and Doyle (1990). Phytoplasma detection was performed by PCR amplification of 16S rRNA gene using the

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Fig. 1 Phytoplasmas affecting plum plants. a: Plum plant infected with PYWB phytoplasma showing symptoms of yellowing and witches' broom, b: TEM image of ultrathin section showing phloem cell with phytoplasma-like pleomorphic bodies (black triangles) obtained from PYWB PCR positive plum sample (scale bar = 2um), cw: cell-wal and c: detail showing the symptoms of a witches´ broom and the decrease size of leaves

primer pairs P1/P7 (Deng and Hiruki 1991) and R16F2n/R16R2 (Lee et al. 1993) in direct and nested PCR reactions respectively (Fernández et al. 2013). Also, ultrathin sections of leaf midrib sampled on symptomatic plants were observed using Transmission Electron Microscopy (TEM) following Nome et al. (2007) protocol. PCR -RFLP patterns of 1.2 kb amplicons using *Hha*I, *Rsa*I and *Tru*1I (*Mse*I) enzymes were analyzed by electrophoresis in agarose:Methapore™ gels (1.5%:0.5% *w/v*), stained with GelRed® and visualized under UV light. Partial sequences of 16S rRNA gene were obtained by cloning PCR amplifications (primers R16F2n/R16R2) in pGEM-T easy Vector System I (Promega, USA) following the manufacturer's recommendations. From each sample three clones were sequenced from both extremes (T7-SP6

Table 1 RFLP similarity coefficient values (F) of 16SrXIII (Mexican periwinkle virescence, MPV) subgroups. A-K: Reference pattern for subgroup A (AF248960), B (U96614), C (AF495882), D (FJ914647), E (JQ79217), F (KJ921641), G (KU850940), H (JX626329), I (KU896186), J (EU719108), K (MH939194) and L*: New subgroup proposed in this paper (MT553105)

16SrXIII-subgroups	A	B	C	D	E	F	G	H	I	J	K	L*
A	1											
B	0.89	1										
C	0.87	0.81	1									
D	0.91	0.9	0.82	1								
E	0.92	0.87	0.83	0.89	1							
F	0.89	0.78	0.76	0.8	0.82	1						
G	0.91	0.86	0.95	0.86	0.83	0.8	1					
H	0.95	0.9	0.91	0.9	0.91	0.84	0.92	1				
I	0.92	0.95	0.84	0.93	0.9	0.81	0.89	0.93	1			
J	0.58	0.76	0.5	0.53	0.54	0.65	0.53	0.55	0.53	1		
K	0.91	0.84	0.78	0.86	0.87	0.84	0.82	0.86	0.89	0.55	1	
L*	**0.94**	**0.83**	**0.92**	**0.85**	**0.87**	**0.83**	**0.97**	**0.89**	**0.86**	**0.54**	**0.85**	**1**

primers) using commercial services (Macrogen, Korea). Final consensus sequences were assembled using Geneious vR.10 software and deposited in Genbank. The 16Sr DNA group-subgroup affiliations were assigned by in silico RFLP profile using *i*PhyClassifier program (Zhao et al. 2009). Multiple alignments were conducted using MAFFT and phylogenetic relationships were inferred applying the Maximum Likelihood method (bootstrap 1000) using the software MEGA 7 (Kumar et al. 2016).

Nested PCR amplifications were obtained in 4/5 symptomatic plum and 3/3 symptomatic peach samples. No amplifications were observed from asymptomatic DNA samples. Also, the presence of pleomorphic bodies within phloem cells were detected by TEM in 2 PCR positive plum samples (Fig. 1b). The PCR -RFLP analysis showed the presence of two different profiles. Tree plum samples (Plum Yellows Witches´ Broom: PYWB-ArgG1, PYWB-ArgG2 and PYWB-ArgP1) and the tree peach samples (data not show) shared an identical profile within the '*Ca*. Phytoplasma pruni' (16SrIII-B subgroup) for *Tru*1L, *Rsa*I and *Hha*I nucleases. These results were also confirmed by the *i*PhyClassifier since 16S rDNA gene sequences of these samples (MT553102, MT553103 and MT553104) showed an identical RFLP pattern for the 17 enzymes evaluated (similarity coefficient F = 1) with the representative pattern of subgroup 16SrIII-B (AF189288). Nucleotide similarity analysis conducted with BLASTn Program (http://www.ncbi.nlm.nih.gov/BLAST) showed identity values >99% within X-disease group (16SrIII) phytoplasmas sequences. On the other hand, PYWB-ArgP3 sample showed an actual RFLP-profile similar to those described for the China tree yellows phytoplasma (subgroup 16SrXIII-G) (Fernández et al. 2016). In silico RFLP of

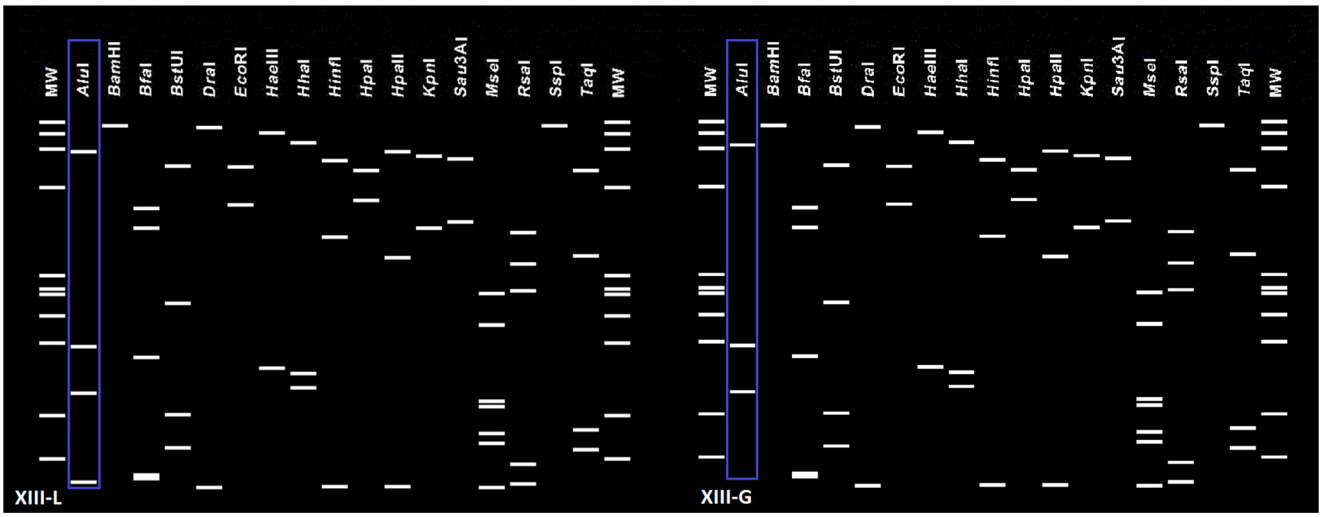

Fig. 2 Comparison of virtual RFLP-profile between subgroups 16SrXIII-G and novel 16SrXIII-L using *i*PhyClassifier. XIII-L: collective RFLP profile of PYWB-ArgP3 (MT553105), XIII-G: collective RFLP profile of ChTYXIII (DQ444264), MW: MW: ϕ X174-HaeIII digest, in blue box the differential pattern of the *Alu*I enzyme is highlighted

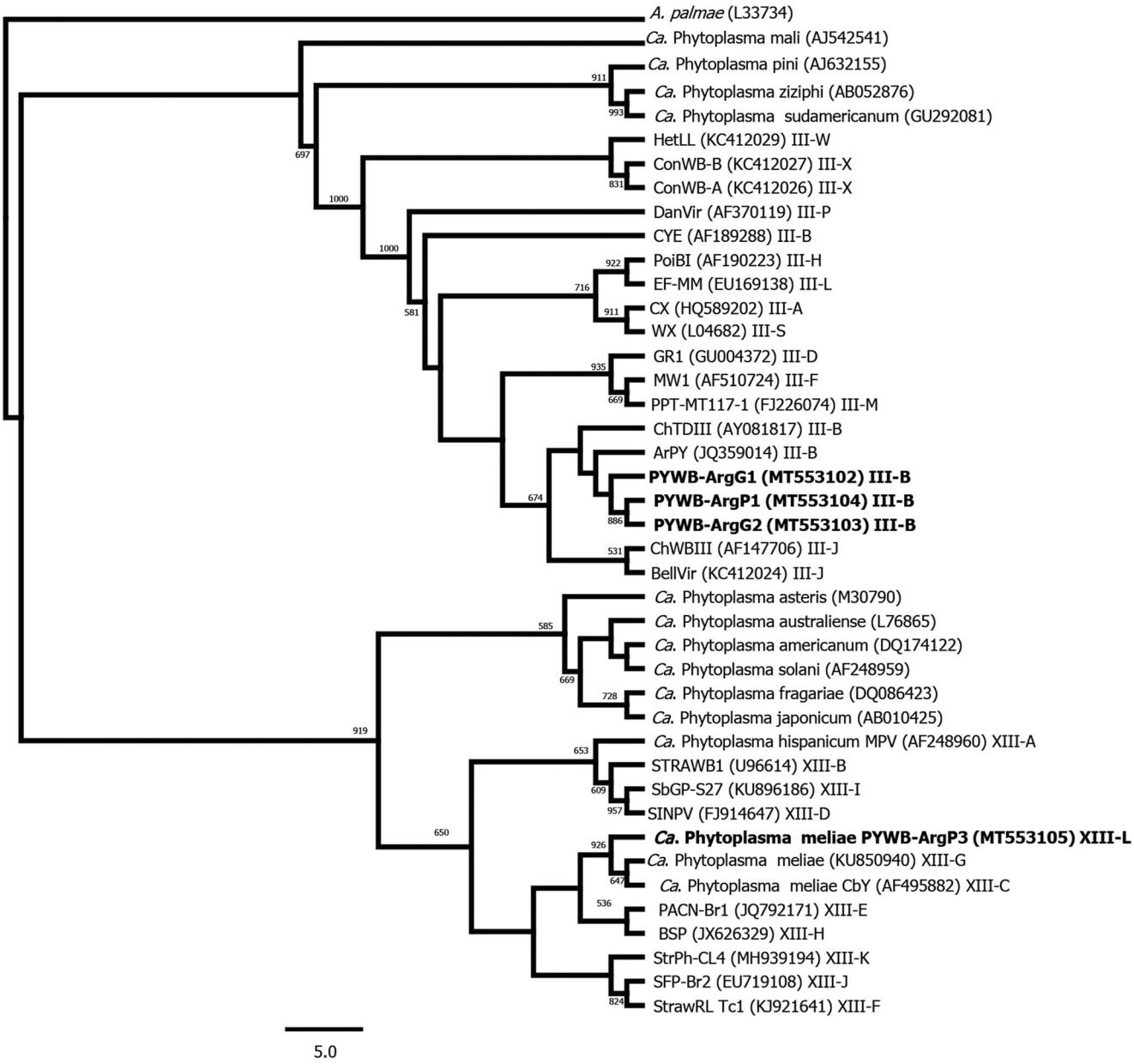

Fig. 3 Phylogenetic tree inferred from analysis of 16S rRNA sequences by the Maximum Likelihood method. The GenBank accession numbers for each taxon are given in parentheses. The corresponding 16SrIII and 16SrXIII-subgroups are after each taxon. The numbers on the branches are bootstrap (confidence) values (1000 replicates). Plum Yellows Witches' Broom (PYWB) sequences obtained in this work are in bold. *Acholeplasma palmae* was used as outgroup. The scale bar represents the number of nucleotide substitutions per site

PYWB-ArgP3 sequence (MT553105) showed that the most similar profiles are those belonging to subgroup 16SrXIII-G (DQ444264) (F = 0.97) (Table 1, Fig. 2).). As PYWB -ArgP3 shares an identity of 99.70% whit the 16S rDNA gene sequence of '*Ca*. Phytoplasma meliae' reference strain (KU850940), it can be considered as a related strain. The phylogenetic tree was consistent whit the RFLP results and nucleotide identity analysis as PYWB-ArgG1, ArgG2 and ArgP1 were grouped within the representative sequences of subgroup 16SrIII-B while PYWB-ArgP3 was clustered within the 16SrXIII-clade but as external node from subgroups 16SrXIII-G/C (Fig. 3). As far as we know, this is the first report of phytoplasmas in association with yellowing plum in Argentina. Subgroup 16SrIII-B is one of the most cited phytoplasmas in South America affecting diverse plant species (Galdeano et al. 2013). Interestingly, the presence of phytoplasmas from this subgroup in association with peaches has been previously reported in the same area where the plum samples were collected (Fernández et al. 2013). Although potential vector insects have been identified for phytoplasmas of the X-disease group in the region (Eckstein et al. 2013; Kreyci et al. 2017), further investigations are necessary to define the

role that these insects play in the spread of the disease. In this work, plum is showed as new host for the '*Ca*. Phytoplasma meliae', which was only reported affecting chinaberry trees in Argentina and Bolivia (Harrison et al. 2003;Fernández et al. 2016). Despite the high identity values, PYWB-ArgP3 has a unique RFLP profile which differ from all 16SrXIII-subgroups described so far. The last subgroup 16SrXIII-K was described in association to strawberry phyllody in Chile (Cui et al. 2019). Following the traditional nomenclature in the assignment of letters for the new subgroups, we propose that the PYWB-ArgP3 have be considered as reference sequence for the novel subgroup 16SrXIII-L. The results presented here contribute to a better understanding of phytoplasma diversity in South America, especially in the *Prunus* genera, and highlight the need to continue investigating the role of insects in the dispersal of this class of pathogens in the region.

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