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Detection

New phytoplasma subgroups within the 16SrI and 16SrVII groups detected in trees of Bogotá, Colombia

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Abstract

Phytoplasmas of groups 16SrI and 16SrVII are associated with a disease in urban trees in Bogotá, Colombia. The objective of this work was to study the diversity of these phytoplasmas, so 16S rRNA amplicons from the two groups were cloned and sequenced. Using the *i*Phyclassifier tool, sequences of 18 clones were assigned to subgroups 16SrI-B, and to three news subgroups with a restriction pattern similar to that of 16SrI-B and to that of 16SrI-K phytoplasmas. Phytoplasmas of the 16SrVII group were tentatively assigned to a new subgroup with a restriction pattern similar to that of the 16SrVII-A. These results suggest that the phytoplasmas that presently infecting the trees of Bogotà show great genetic variability probably indicating their divergence from original strains from other crops in Colombia such as corn (16SrI-B) and ash (16SrVII-A).

Keywords: ribosomal subgroups, phytoplasma diversity, molecular identification

Introduction

Phytoplasmas are plant pathogenic bacteria of the class *Mollicutes* that lack cell walls, are sensitive to tetracycline and are obligate parasites of the phloem of infected plants and of the tissues of Hemipteran insects. They are transmitted by sap feeder insect species of the Cicadellidae, Cixiidae, Psyllidae, Cercopidae and Delphacidae families, vegetative propagation, grafting and in some cases by seeds. They have small genomes of 600 to 1,600 kb, with low G+C contents (Bertaccini and Duduk, 2009). In the Sabana de Bogotá, Colombia, '*Candidatus* Phytoplasma asteris' (16SrI) and '*Candidatus* Phytoplasma fraxini' (16SrVII) are the major phytoplasma ribosomal groups that infect urban trees. Symptoms such as yellowing and distortions of crown shape and growth patterns are common in *Populus nigra* (Salicaceae), and *Quercus humboldtii* (Fagaceae) infected with phytoplasmas (Franco-Lara and Perilla-Henao, 2014). The objective of this work was to study the genetic variability of the phytoplasmas in the groups 16SrI and 16SrVII infecting the trees of Bogotà using *P. nigra* and *Q. humboldtii* as study models.

Materials and Methods

Samples were collected from 11 *P. nigra* (Pn) and 23 *Q. humboldtii* (Qh) trees. DNA was extracted and used in amplification assays. The 16S rRNA gene was amplified with primers R16mF2/R16mR1 followed in nested PCR by R16F2n/R16R2 (Gundersen and Lee, 1996). The latter amplicons were analyzed by RFLP with restriction enzymes *Alu*I, *Hha*I, *Mse*I, and *Rsa*I, or by sequencing. Amplicons from selected strains were cloned with the pMiniT™ 2.0 Vectors® (NEB) Kit. DNA of the maize bushy stunt phytoplasma (MBS) (16SrI-B) from Colombia or from the ash yellows phytoplasma (ASHY) (16SrVII-A) from North America were used as positive controls, and water as negative control. PCR assays were conducted in a final volume of 15 µl with 0.05 U/µl of *Taq* Biolase, 1 X reaction buffer, 0.2 mM dNTPs (Bioline®), 0.2 µM primers, and 20–50 ng of template DNA. The thermal profile for amplifications was: initial melting for 10 minutes at 94°C followed by 35 cycles at 94°C for 1 minute, 54°C for 2 minutes and 72°C for 2 minutes with a final extension for 10 minutes. The amplicons were detected in a 1% agarose gel after electrophoresis and RFLP products were separated in a 3% agarose gel. The sequencing reactions were performed by Macrogen, Korea. Forward and reverse sequences were edited with Geneious 9.1.4. and consensus sequences were compared with the BLASTn tool to GenBank database, then analyzed with the *i*PhyClassifier (Zhao *et al*., 2009).

Results

Amplicons of the expected sizes were obtained from 11 Pn, and 14 Qh trees. In both tree species phytoplasmas classified in the 16SrI and 16SrVII groups were detected and no mixed infection was observed (Table 1 and Figure 1). The sequencing analysis confirmed the identity of the phytoplasmas. Since

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the quality and length of the sequences obtained by PCR was not good enough for the *i*Phyclassifier analysis, selected amplicons obtained in nested reactions were cloned and sequenced, as well as the positive ASHY and MBS positive controls. Three clones per sample were sequenced and high quality sequences were obtained for 18 clones. Sequences from the same clone were not identical, with similarities ranging between 99% and 99.3%. Sequences from Pn9, Pn10, Pn11, PnQ12, and Qh22 clustered to phytoplasmas in group 16SrI and sequences Pn3, Pn6, and Qh6 to group 16SrVII. Using the *i*PhyClassifier tool the sequences belonging to the 16SrI group were classified into i) subgroup 16SrI-B (samples Pn9-2, Pn10-1, Pn10-3, Qh22-1); ii) a subgroup named 16SrI-AF with a restriction pattern very similar to that of 16SrI-B (samples Pn9-3, Pn-2, Qh12-1, Qh12-2, Qh12-3); iii) a new subgroup referred here as 16SrI-AH with a restriction pattern very similar to that of subgroup 16SrI-K (sample Pn11-1); and iv) another new subgroup referred here as 16SrI-AG with a restriction pattern very similar to that of 16SrI-AC (sample P9-1). For the 16SrVII sequences the restriction patterns corresponded to a new subgroup 16SrVII-G, very similar to that of 16SrVII-A (samples Pn3-1, Pn3-2, Pn3-3, Pn6-1, Pn6-2, Qh6-2, Qh6-3). All the sequences of the MBS corresponded to the 16SrI-B and all those of ASHY to the 16SrVII-A subgroups.

Table 1. Results of the nested PCR and RFLP analysis, and number of sequences obtained from the cloning of the 16S rRNA gene.

	Number of PCR positives[1]	RFLP		Sequencing	
		16SrI	16SrVII	'*Ca*. P. asteris'	'*Ca*. *P. fraxini*'
P. nigra	11/11	5	6	2	5
Q. humboldtii	14/16[2]	3	11	4	8
Total	27	8	17	6	13

[1]Numbers of positives of all trees tested in nested PCR analyses.
[2]Samples producing faint bands and not analyzed using RFLP.

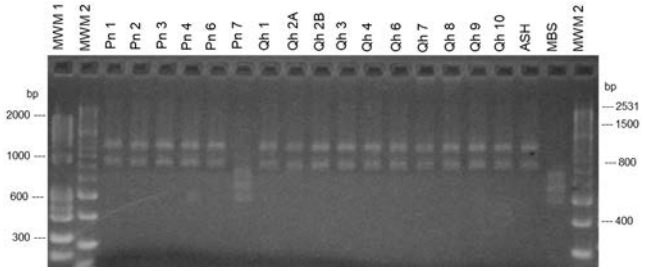

Figure 1. RFLP analysis with *Mse*I of nested amplicons of *P. nigra* (Pn) and *Q. humboldtii* (Qh). ASHY and MBS, positive controls. MWM 1, Hyper ladder 50 bp; MWM 2, Hyper ladder 25 bp (Bioline).

Discussion

The objective of this work was to determine the phytoplasma subgroups that infect *P. nigra* and *Q. humboldtii* in Bogotá. No clones from the same amplicon produced identical sequences, with similarities between 99% and 99.3%, which suggest that the trees are infected with mixtures of very similar phytoplasmas. For example, in sample Pn9, the three clones examined were classified in different subgroups within the 16SrI group *i.e.* 16SrI-B and three subgroups designed 16SrI-AF, 16SrI-AG and 16SrI-AH. A further subgroup 16SrI-AC had been previously reported in Bogotá by verification of GenBank available sequences (Pérez-López *et al*., 2016). Moreover, all the phytoplasmas assigned to the 16SrVII group infecting both tree species, belonged to the new subgroup 16SrVII-G. These results confirm the high diversity of the phytoplasmas infecting the trees of Bogotà particularly of those classified in group 16SrI and suggest that these strains belong to a diversified population as has been suggested for other phytoplasmas (Davis *et al*., 2018). Moreover this aspect was already reported in Europe in poplar trees and in North America in ash trees (Griffiths *et al*., 1999; Seruga *et al*., 2003).

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