Molecular characterization of a '*Candidatus Phytoplasma asteris*'-related strain (16SrI-B / cpn60UT I-IB) affecting daisies (*Argyranthemum* spp.) in Mexico

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Abstract

From 2018 to 2019 symptoms consistent with infection by plant pathogenic phytoplasma bacteria were observed in commercial flower greenhouses located in Atlixco, Puebla, Mexico. Two varieties of *Argyranthemum* spp. plants exhibited symptoms of phyllody, causing serious concerns among farmers. To determine if phytoplasmas were associated with the symptomatic plants, the 16S rRNA-encoding locus (F2nR2 fragment) was amplified by PCR. This revealed that the symptomatic plants collected were infected by a phytoplasma belonging to the aster yellows group (16SrI-B). These results were confirmed and refined by amplification and sequencing of the phytoplasma chaperonin 60 universal target (*cpn60* UT). Sequence analysis using the online tool CpnClassiPhyR confirmed that the phytoplasma detected is a member of the 16SrI group, specifically a member of the *cpn60* UT I-IB subgroup.. This is the first report of daisies affected by phytoplasma in Mexico, and the first report of the use of CpnClassiPhyR to characterize an unknown phytoplasma strain. The detection of phytoplasmas infecting commercially produced daisies and their characterization provides an impetus for phytoplasma management in Atlixco, a Mexican city for which the production of ornamental plants and cut flowers is of economic importance.

Keywords Aster yellows phytoplasma · Flowers · 16SrI-B · Atlixco · CpnClassiPhyR

Phytoplasmas ('*Candidatus Phytoplasma*' spp.) are insect-transmitted bacteria limited to the phloem and have been associated with a wide array of diseases in many ornamental and food crop plants (Pérez-López et al. 2016a). Daisies, chrysanthemums, and other flowers within the family Asteraceae are susceptible to phytoplasma infection

and were among the first phytoplasma hosts described (Lee et al. 2000). Such is the importance of these plant hosts that the first phytoplasma taxonomic group ever described (16SrI), was named 'aster yellows' group (Lee et al. 1993, 1998, 2000), and given the provisional species name '*Candidatus phytoplasma asteris*' (Lee et al. 2004), reflecting their unculturable nature. Aster yellows phytoplasma affects more than 300 plant species, primarily in the aster family, but also many horticultural, oilseed and cereal crops have been affected by this phytoplasma group (Kumari et al. 2019).

Argyranthemum spp. are ornamental flowers in the family Asteraceae that are susceptible to phytoplasma infection and commonly known as daisies in Mexico, although formally daisies are *Bellis perennis*. These plants, which are colloquially called daisies among growers, display symptoms on infection that include phyllody, in which floral tissues become leaf-like, and are therefore easily recognized by their green flowers (Lee et al. 2000). Daisy plants have been observed to be affected by many different phytoplasma strains belonging to the ribosomal groups

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16SrI (Lee et al. 2003, D'Amelio et al. 2008, 2010), 16SrIII (Galdeano et al. 2013), and 16SrVI (Hemmati et al. 2018), among others. In Mexico, in the states of Puebla, Michoacán and Puebla, daisies were reported to be associated with 16SrI phytoplasma infections, but in that case the plants were *Dimorphotheca sinuate* (Ortega-Acosta et al. 2019). Phytoplasma strains belonging to the aster yellows group ('*Candidatus Phytoplasma asteris*') have been reported in Mexico affecting other ornamental plants such as marigold (*Tagetes erecta*) (Rojas-Martínez et al. 2003) and periwinkle (*Vinca minor*) (Pérez-López et al. 2016c).

Mexico is the third largest producer of ornamental plants worldwide, and in Mexico, the state of Puebla is one of the top five producers of ornamental plants (Notimex 2019). Atlixco, particularly, is a city economically well known in Puebla for its production of ornamental plants and cut flowers, and for this reason the detection of symptomatic flowers was a concern to farmers from the area. To determine the possible cause of the observed symptoms in daisies, we amplified and sequenced the phytoplasma taxonomic markers 16S rRNA (F2nR2) and chaperonin 60 universal target (*cpn60* UT) from symptomatic plants. The *cpn60* UT has been used to characterize phytoplasma strains to such an extent that recently an online freely available tool called CpnClassiPhyR (<http://cpnclassiphyr.ca>) was developed and validated (Muirhead et al. 2019a, b). Using this tool, users can determine if a query *cpn60* sequence belongs to a phytoplasma and obtain the restriction fragment length polymorphism (RFLP)-based *cpn60* UT classification of the unknown phytoplasma strain (Pérez-López et al. 2016a). Users can also generate the *in silico* RFLP gel and a phylogenetic tree to place the query sequence into the context of all phytoplasmas with known *cpn60* UT sequences. This study is the first report of *Argyranthemum* spp. plants affected by phytoplasmas in Mexico, and represents the first practical application of the online tool CpnClassiPhyR. The results obtained using *cpn60* UT were consistent with the analysis of the 16S rRNA-operon, and provided further taxonomic information for these strains.

In January 2019, three daisy (*Argyranthemum* spp.) plants belonging to two different varieties exhibiting phyllody (AgP1 to AgP3) (Fig. 1a) were collected from Atlixco, Puebla (Fig. 1b), Mexico, along with three asymptomatic and presumably healthy daisy plants. Each pair of plants (symptomatic and asymptomatic) was collected from a different greenhouse but located in the same row. Leaf and peduncle samples were collected from all symptomatic and asymptomatic plants. Total DNA was extracted from 0.3 g of each sample as described previously (Pérez-López et al. 2016b). The phytoplasma *cpn60* UT was amplified using polymerase chain reaction (PCR) using phytoplasma universal primers as previously

described (Dumonceaux et al. 2014). To corroborate the results obtained based on *cpn60* UT, the 16S rRNA-encoding gene was amplified using primer pair P1/P7 (Deng and Hiruki 1991; Schneider et al. 1995) followed by primer pair R16mF2/R16mR2 (Lee et al. 1993) in a subsequent nested PCR to amplify the F2nR2 fragment of the 16S rRNA-encoding locus. The secondary PCR used 2 µl of a 1:30 dilution of the product of the primary PCR as template, and PCR conditions for both rounds were as described (Smart et al. 1996).

The 604 bp *cpn60* UT and 1.5 kb F2nR2 amplicons generated from the symptomatic plants were purified using a QIAquick PCR Purification Kit (Qiagen) and sequenced with primers H279p/H280p:D0317/D0318 (1:7 ratio) and R16mF2/R16mR2, respectively (Macrogen). Contigs were assembled using the Staden package (Bonfield and Whitwham 2010) and examined using BLAST (<http://www.ncbi.nlm.nih.gov>) to determine the closest matches to reference sequences. The 16S rRNA-encoding gene-based F2nR2 sequence was analyzed using the on-line tool iPhyClassifier (Zhao et al. 2009) to determine the group and subgroup classifications and corroborate observations made using *cpn60* UT. 16S sequences were subjected to phylogenetic analysis using the maximum likelihood method with MEGA v6.0 (Tamura et al. 2013), using 1000 bootstrap repetitions. The *cpn60* UT sequences were analyzed using the on-line interactive tool CpnClassiPhyR (Muirhead et al. 2019a, b) (<http://cpnclassiphyr.ca>) to determine the *cpn60* UT-based group and subgroup classifications of the strains. The virtual RFLP pattern obtained from *cpn60* UT DNA sequences was compared with the RFLP patterns from previously identified strains. Automated phylogenetic analysis was performed by the CpnClassiPhyR and corroborated manually using the methods described above. *Acholeplasma laidlawii* strain PG-8AT (U14905) was used as an outgroup for both automatically and manually generated trees.

Phyllody was observed in daisy plants (Fig. 1a, Fig. S1) in Atlixco, Puebla (Fig. 1b). Template DNA extracted from symptomatic plant AgP2 yielded the approximately 1500 bp F2nR2 amplicon, with controls using DNA from asymptomatic plants, along with no template controls, failing to yield amplification products. The F2nR2 sequence obtained from the three plant hosts was 99.6% identical to the reference strain of '*Candidatus Phytoplasma asteris*' (GenBank accession no. M30790). We deposited the sequence into GenBank (AgP2, MW293910) and the phylogenetic analysis performed confirmed that the strains belong to the 16SrI ribosomal group (Fig. 2c), branching mainly with members of the 16SrI-B subgroup. To confirm the subgroup identity, we submitted the F2nR2 sequence to iPhyClassifier (<https://plantpathology.ba.ars.usda.gov/cgi-bin/resource/iphyclassifier.cgi>) and the *in silico* RFLP

Fig. 1 Symptoms and location of sampling area of phytoplasma infected samples. **a** Phyllody displayed by the daisy plants collected in this study with AgP1 to AgP3 from top to bottom. Left panel,

symptomatic and right panel, asymptomatic. AgP 1 and 3 belong to the same variety. **b** Location of the symptomatic samples collected. Map of Puebla, Mexico with the precise localization of Atlixco

pattern obtained was identical to the 16SrI-B reference strain OY-M (GenBank accession no. AP006628) with $F = 1.00$ (Fig. 2d).

Template DNA extracted from symptomatic plants also yielded an approximately 600 bp *cpn60* UT amplicon. Controls featuring DNA from uninfected (asymptomatic) plants, as well as no template controls, failed to yield amplification products. Results from BLAST showed that the *cpn60* UT sequences obtained from the three plant hosts were 100% identical to one another and to the reference strain of '*Candidatus Phytoplasma asteris*' subgroup *cpn60UT* I-IB (Strain SF1, GenBank accession no. KJ940013). This result was confirmed through phylogenetic analysis showing that the *cpn60* UT sequences amplified from the positive *Argyranthemum* spp. samples (GenBank accession no. AgP1-MN893667 to AgP3-MN893669) branched again with strains in the *cpn60* UT I-IB subgroup (Fig. 2a). The *in silico* RFLP pattern obtained for AgP2 using CpnClassiPhyR

(<http://cpnclassiphyr.ca>) was also identical to the pattern for the strain SF1 with $F = 1.00$ (Fig. 2b).

To date, the 16SrI group is the most diverse phytoplasma group with thirty-three subgroups, 16SrI-A to 16SrI-AI (Pérez-López et al. 2019a). Among these, subgroup 16SrI-B is the most widely distributed geographically but also affects the widest array of plant hosts (Pérez-López et al. 2016a; Muirhead et al. 2019a, b). In Mexico, 16SrI-B subgroup has been previously reported to affect *Dimorphotheca sinuate* daisies (Ortega-Acosta et al. 2019), marigold (Rojas-Martínez et al. 2003), *Amaranth* spp. (Rojas-Martínez et al. 2009), citrus (Arratia-Castro et al. 2014), maize (Pérez-López et al. 2016b), periwinkle (Pérez-López et al. 2016c), grass (Pérez-López and Dumonceaux 2018), and sugarcane (Pérez-López et al. 2019b). Here, we expanded the hosts for 16SrI-B in Mexico to include *Argyranthemum* spp. (daisies), which is a very important commodity for the Mexican economy.

Fig. 2 Identification of AgP strain as a member of the 16SrI-B and *cpn60UT* I-IB. **a** Phylogenetic relationship inferred from analysis using maximum likelihood of *cpn60UT* sequences obtained in this study and phytoplasma strains from different ribosomal groups and subgroups. Corresponding *cpn60UT* groups and subgroups as well as GenBank accession numbers between parentheses. Strains within *cpn60UT* I-B subgroups are tagged with white circles, while AgP strains were tagged with black circles. Bar, 20 nucleotides substitution. **b** Computer simulated RFLP analysis of the *cpn60UT* of AgP2 with the seven endonucleases enzymes defined by Pérez-López et al. (2016d). **c** Phylogenetic relationship inferred from analysis using maximum likelihood of F2nR2 sequence obtained in this study and phytoplasma strains from different ribosomal groups and subgroups. Corresponding 16Sr groups and subgroups as well as GenBank accession number between parentheses. Strains within the 16SrI-B subgroup were tagged with white circles while AgP strain was tagged with a black circle. Bar, 50 nucleotides substitution. **d** Computer simulated RFLP analysis of the F2nR2 of AgP with the seventeen endonucleases enzymes defined by Lee et al. (1998)

Phytoplasma classification and taxonomy based on 16S rRNA-encoding gene sequences has proven to be very useful but there are limitations associated with their exclusive use, such as high DNA sequence identity among closely related taxa, limiting the resolution of closely related but distinct strains, and the presence of two copies of the 16S rRNA-encoding gene that can be distinct, a phenomenon known as heterogeneity (Wei et al. 2007; Zhao et al. 2009; Pérez-López and Dumonceaux 2016). These are the main reasons why single-copy, protein-coding genes have been explored to characterize phytoplasmas (Lee et al. 2006; Martini et al. 2007; Makarova et al. 2012; Valiunas et al. 2013). The chaperonin-60 gene (*cpn60*, with synonyms

groEL and *hsp60*), which encodes a 60 kDa protein, specifically a subsequence of *cpn60* corresponding to nucleotides 274–828 of the *E. coli cpn60* gene, has been identified as a suitable barcode marker for the Domain Bacteria (Links et al. 2012). This molecular marker has been successfully used to characterize phytoplasmas, including the development of a classification scheme that allows a finer differentiation of phytoplasma strains within the same 16S rRNA RFLP subgroup (Pérez-López et al. 2016d). This study is the first application of CpnClassiPhyR to identify and characterize unknown phytoplasma strains affecting naturally infected plants. We also found correspondence between the *cpn60UT* classification and the classification

based on the 16S rRNA-encoding gene segment F2nR2 using the complementary *iPhyClassifier* tool, which supports the suitability of CpnClassiPhyR as a tool to study phytoplasmas.

The identification of new plant hosts susceptible to phytoplasmas is very important for informing producers' decisions regarding effective management strategies. A common practice among farmers in Atlixco, Puebla is the prompt elimination of flowers showing phyllody. After the present study, the elimination of symptomatic plants has been dispersed among farmers and greenhouse managers. In this study we report for the first time the presence of phytoplasmas affecting *Argyranthemum* spp. plants in Mexico, and we have also expanded the known host range for 16SrI-B, a subgroup that is proving to be ubiquitous in Mexico. The identification of the insect vector(s) responsible for the dissemination of the phytoplasma strain is ongoing.

Supplementary Information The online version contains supplementary material available at <https://doi.org/10.1007/s13314-020-00415-7>.

Compliance with ethical standards

Competing Interest The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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