SCIENTIFIC NOTE

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Identification of a phytoplasma associated with witches' broom symptoms in calafate (*Berberis microphylla* G. Forst.)

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ABSTRACT

Berberis microphylla G. Forst. ("calafate") is a native berry grown in the Patagonian area of Chile and Argentina. The calafate fruit have important commercial projection, given that contains antioxidant characteristics with high levels of phenolic compounds and anthocyanins. Recently, wild growth of calafate present in Region of Magallanes y la Antártica Chilena has been affected by symptoms of witch's broom, which is associated to infections caused by phytoplasmas. To determine the presence of phytoplasmas in calafate plants affected by these symptoms, symptomatic and asymptomatic samples were analyzed. To identify the phytopathogen, nested polymerase chain reaction (nested-PCR) was used with three pairs of primers (P1/P7, R16mF2/mR1, R16F2n/R2); sequencing of the fragments obtained from PCR and an *in silico* restriction analysis was carried out on the obtained genomic sequences. Results from nested-PCR showed an expected band of 1.2 kb only in the samples with symptoms. The nucleotide sequence obtained from the amplified DNA fragment had similarity to 99% of other phytoplasmas present in the National Center for Biotechnology Information (NCBI) database, according to a basic local alignment search tool (BLAST) analysis. Restriction analysis indicated that the amplified DNA fragment corresponded to a phytoplasma from the 16SrXIII-f group. In conclusion, the symptoms of witch's broom present in the analyzed calafate plants from Magallanes region signal the presence of the phytoplasma "*Candidatus* Phytoplasma hispanicum" pertaining to the group 16SrXIII-F.

Key words: Calafate, phytoplasmas, witch's broom.

INTRODUCTION

Berberis microphylla G. Forst. ("calafate") belongs to the family Berberidaceae, is a shrub native to the Chilean and Argentinean Patagonia (Varas et al., 2013). The calafate fruits are dark purple, black, or bluish berries and they have an elevated antioxidant capacity, given its high content of polyphenols and anthocyanins (Ruiz et al., 2010). The production of its fruit as a wild crop is concentrated in small gardens in the Region Aysén del General Carlos Ibáñez del Campo and Region Magallanes y la Antártica Chilena for use in jams and juices (Mariangel et al., 2013).

In the last years, calafate plants in these regions have shown symptoms of witch's broom, including proliferation of shoots with short internodes. These symptoms are commonly associated to the presence of phytoplasmas that are prokaryotes from the Mollicutes class (Camarena Gutiérrez and De La Torre Almaraz, 2008; Bertaccini et al., 2014). The phytoplasmas inhabit the sieve tubes of the plant tissue, and move to other plant organs through pores in these cells. These phytoparasites cause diseases associated to symptoms that suggest an imbalance in the processes of accumulation of reserve substances, hormonal balance and photosynthesis and causes symptoms such as yellowing, witch's broom, virescence, phyllody, premature reddening of the leaves, dwarfism and proliferation of adventitious buds, sterility of

flowers, among others, which can cause the death of the host. For survival and permanence in a cultivar, phytoplasmas require insect vectors through which they disseminate and propagate persistently. This means that once acquired by an insect, they constantly multiply and maintain within the vector, making the insect vector potentially infective for the rest of its life (Arismendi et al., 2010b). The literature describes phytoplasma vectors belonging to the families Cicadellidae, Cixiidae, Psyllidae, Delphacidae and Derbidae (Camarena Gutiérrez and De La Torre Almaraz, 2008).

Considering that calafate is an important culinary species, used in the production of jams and juices in the Patagonia, and that the phytoplasmas can cause sterility of flowers and therefore decrease fruit production, the objective of this research was to identify phytoplasma associated to the symptomatology of witch's broom observed in wild plants from Magallanes region, Chile.

MATERIALS AND METHODS

Plant material

To isolate phytoplasma, genomic DNA was extracted from five samples of *Berberis microphylla* G. Forst. All samples were collected in summer 2016 from adult plants located around Fuerte Bulnes near to Punta Arenas city (53°37'S, 70°55' W), three plants showed symptoms of witches' broom and two were asymptomatic (Figure 1). The samples consisted in stem and petioles from young branches, which were placed in plastic bags and correctly labeled. The samples were kept under cold conditions during the transfer to the virology laboratory of Instituto de Investigaciones Agropecuarias (INIA). The DNA was extracted and stored at -80 °C for the polymerase chain reaction (PCR) and sequencing performance.

Extraction of nucleic acids and PCR amplification

From each sample, an enriched phytoplasma DNA extraction was taken, following the protocol described by Ahrens and Seemüller (1992). DNA quantification was carried out with a UV/Vis micro-volume spectrophotometer (ASP-2680, ACTGene, Piscataway, New Jersey, USA). Extracted and quantified DNA was used as a template to amplify a genomic fragment corresponding to the gene 16Sr RNA through nested PCR using three pairs of primers described in the literature (Table 1). These primers were P1/P7, that magnified a region of 1.7 kb which corresponds to position 1 of the 16S rRNA gene and 1784 of 23S gene; R16mF2/mR1, that magnified a region of 1.4 kb, which corresponds to position 48 and 1464 of the 16S rRNA gene and R16F2n/R2; that magnified a region of 1.2 kb which corresponds to position 1279 to 1376 of

Figure 1. Symptoms in calafate. A: Calafate plant with symptoms of witch's broom, indicated by a white arrow. B and C: Calafate plant branches with and without symptoms of witches' brooms (s: with symptoms; a: without symptoms).

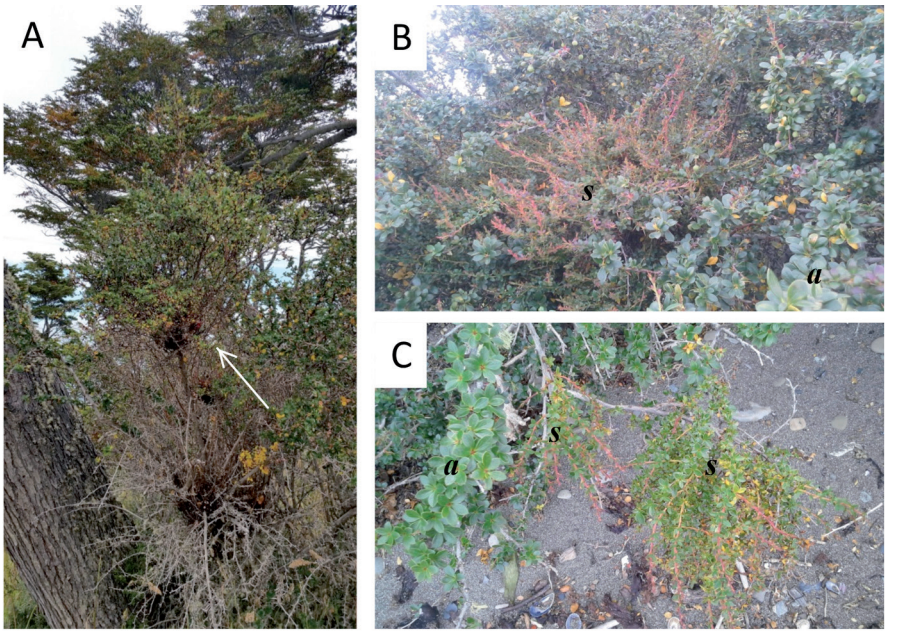


Table 1. Identification, sequence and expected fragment size of the primers used in the amplification of a fragment of the phytoplasma genome.

Id primer	Sequence	Amplified fragment size
P1	5' aagagtttgatcctggctcaggatt 3'	1.7 kb
P7	5' cgtccttcatcggctctt 3'	
R16mF2	5' catgcaagtcgaacgga 3'	1.4 kb
R16mR1	5' cttaaccccaatcatcgac 3'	
R16F2n	5' gaaacgactgctaagactgg 3'	1.2 kb
R16R2	5' tgacgggcggtgtgtacaaaccccg 3'	

the 16S rRNA gene (Lee et al., 1995; Schneider, 1995; Gundersen and Lee, 1996). The first amplification was carried out with the P1/P7 primers and 20 ng of extracted DNA were used as a template. The second amplification was done with the primers R16mF2/mR1, using as a template 1 µL of a 1:30 dilution of the PCR product obtained in the first amplification. For the third amplification, 1 µL of a 1:30 dilution from the PCR product obtained in the second amplification was used with primers R1F2/R2. The three PCR mix reaction were performed in a volume of 25 µL: 2.5 µL 10X PCR buffer, 1 µL $MgCl_2$ (50 mM), 1 µL dNTPs mix (10 mM), 1.25 µL forward and reverse primers, 0.25 µL DNA polymerase (5 U μL^{-1}; Invitrogen, Carlsbad, California, USA), 17.8 µL molecular biology grade water (Corning Incorporated, Manassas, Virginia, USA). DNA from Apple Proliferation Group Phytoplasma infected plants was used as PCR positive control (catalog number: 08009PC; LOEWE Biochemica GmbH, Sauerlach, Germany) and reaction mixture without DNA was used as negative control.

The thermal profile of the 35 cycler of the PCR was as follows: Denaturation 94 °C for 1 min (3 min for the first cycle); alignment at 50 °C, 60 °C and 55 °C for p1/p7, R16mF2/R16mR1 and R16F2n/R16R2 primer, respectively, during 2 min; elongation at 72 °C for 3 min (10 min for the last cycle). The PCR products obtained, from each amplification, were visualized by 2% (w/v) agarose gel electrophoresis run in TAE 1X buffer (Tris-acetate-EDTA) and stained with 0.1 µL mL^{-1} ethidium bromide. The amplicons were observed and photographed on a UV light trans-illuminator (WGD-30S, DAIHAN Scientific, Seongbuk-gu, Seoul, Korea).

Cloning and sequencing

Amplicons obtained from the third nested PCR amplification were bound to the cloning vector pGEM-T easy (Promega, Madison, Wisconsin, USA) according to standard protocol, to later transform competent DH5α *E. coli* cells. Three colonies were chosen for plasmid DNA extraction with the AxyPrep Plasmid Miniprep" kit (Axygen Biosciences, Union City, California, USA) according to manufactures instructions. The purified plasmid DNA was sent for sequencing at Macrogen (Macrogen, Seoul, Korea) using the M13 primers (M13F-pUC: 5' gttttcccagtcacgac 3'/M13R-pUC: 5' caggaaacagctatgac 3'). Resulting sequences were analyzed using the program CLC Main Workbench V. 5.5 (QIAGEN, Hilden, Germany) and compared with sequences from the National Center for Biotechnology Information database (NCBI, Bethesda, Maryland, USA) through a basic local alignment search tool (BLASTn).

Restriction analysis (RFLP)

Classification of the 16S group of the pathogen was done by *in silico* restriction analysis using a virtual platform from the USDA for phytoplasma classification, *i*PhyClassifier (Zhao et al., 2009). This online tool does a computerized simulation of an restriction fragment length polymorphism (RFLP) analysis based on recognition of a set of 17 restriction enzymes (*Alu*I, *Bam*H1, *Bfa*I, *Bst*UI, *Dra*I, *Eco*RI, *Hae*III, *Hha*I, *Hin*fI, *Hpa*I, *Hpa*II, *Kpn*I, *Sau*3AI, *Mse*I, *Rsa*I, *Ssp*I, *Taq*I). Results from the RFLP analysis were compared with reference patterns from groups and subgroups of known phytoplasmas.

Phylogenetic analysis

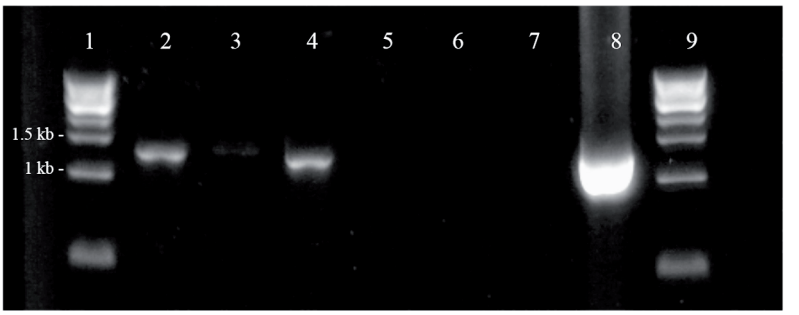
The Chilean phytoplasm isolates were submitted to a multiple alignment with 17 phytoplasma sequences from 16S rRNA gene reported in the NCBI database and a dendrogram was constructed with the MEGA7 software V. 7.0.26 by the Neighbor-Joining method with 500 bootstrap. The evolutionary distance was calculated using the maximum composite likelihood method.

RESULTS AND DISCUSSION

Amplification of a fragment from 16SrRNA gene

Results from amplification of the phytoplasma gene fragment mediated by nested PCR were positive when samples of DNA from calafate were used that manifested symptoms of witch's broom. The final product of the nested PCR was a 1.2 kb band, using the primers P1/P7, R16mF2/mR1 and R16F2n/R2. When DNA templates were used coming from asymptomatic calafate samples, no bands were observed (Figure 2). The nested PCR assay was used to increase sensitivity and specificity of the phytoplasma detection (Duduk and Bertaccini, 2011). The study of 16s DNA polymorphism allows the detection and characterization of phytoplasmas (Bertaccini and Duduk, 2009). The system is based on the amplification of a fragment of the 16S rRNA gene by means of a nested PCR, followed by an RFLP analysis. The success in detecting phytoplasma mediated by PCR depends on obtaining a good quality DNA, because of the total DNA extracted, a few percentage corresponds to phytoplasma DNA (Bertaccini and Duduk, 2009). Then is very important to include a phytoplasma DNA enrichment step in the extraction DNA protocol.

Figure 2. Nested-PCR amplification using primers R16F2n/R2. Row 1: Molecular weight (MW) marker. Rows 2, 3 and 4: calafate samples with witches' brooms symptoms; Rows 5 and 6: calafate samples without witches' broom symptoms; Row 7: negative control (C-); Row 8: positive control (C+); Row 9: MW marker. Amplification of a band of approximately 1.2 kb is seen in samples 1, 2 and 3.



Identification of phytoplasma

The size of the sequence from the DNA fragment corresponding to a band of 1.2 kb from the Chilean calafate isolate was 1244 bp. Upon analysis of this sequence with the online tool *i*PhyClassifier (Zhao et al., 2009), 98.8% similarity to the reference strain *Candidatus* Phytoplasma hispanicum (GenBank: AF248960) was obtained. When these strains were submitted to a multiple alignment with 17 phytoplasma sequences from 16S rRNA gene reported in the NCBI database, a dendrogram was generated that confirmed that the phytoplasma present in calafate belonging to the Mexican periwinkle virescence group 16SrXIII (Figure 3), with 99% identity with Strawberry phylloid fruit phytoplasma, Brazil isolated (GenBank: EU719109) and Strawberry read leaf phytoplasma, Argentina isolated (GenBank: KJ921641). The *in silico* restriction analysis (RFLP) carried out with the online tool *i*PhyClassifier showed a cleavage pattern identical to the reference pattern for the 16SR XIII subgroup F (GenBank: KJ921641) (Figure 4).

The 16SrXIII group is present in region of South America affecting various crops such as potatoes (*Solanum tuberosum* L.), broccoli (*Brassica oleracea* L.), strawberry (*Fragaria ×ananassa* Duchesne ex Rozier) and papaya (*Carica papaya* L.), among others (Santos-Cervantes et al., 2010; Eckstein et al., 2013; Melo et al., 2013; Fernández et al., 2015). Results shown in this study indicate that the group 16SrXIII-F also affects Berberidaceae family, and therefore is added to the number of reports that indicate the presence of phytoplasmas in diverse crops from Chile such as natives plants (Arismendi et al., 2010a) and fruit trees (Gajardo et al., 2009; González et al., 2011). To our knowledge, this is the first report of a phytoplasma affecting Calafate plants in Chile.

Figure 3. Phylogenetic dendrogram constructed using the Neighbor-Joining method. Sequences from the region 16S *r*RNA from 1.2 kb phytoplasma obtained from Chilean calafate isolate (5422 Calafate Chile) and compared with 17 other phytoplasma nucleotide sequences available in the NCBI database. The dendrogram is organized according to the type of phytoplasma, country where it was found, and the group to which it belongs. A bootstrap test of 500 replicates is shown as a percentage together with the nodes. The evolutionary distance was calculated using the maximum composite likelihood method. The final dataset consisted in 1227 positions. Analysis was carried out with MEGA7 software.

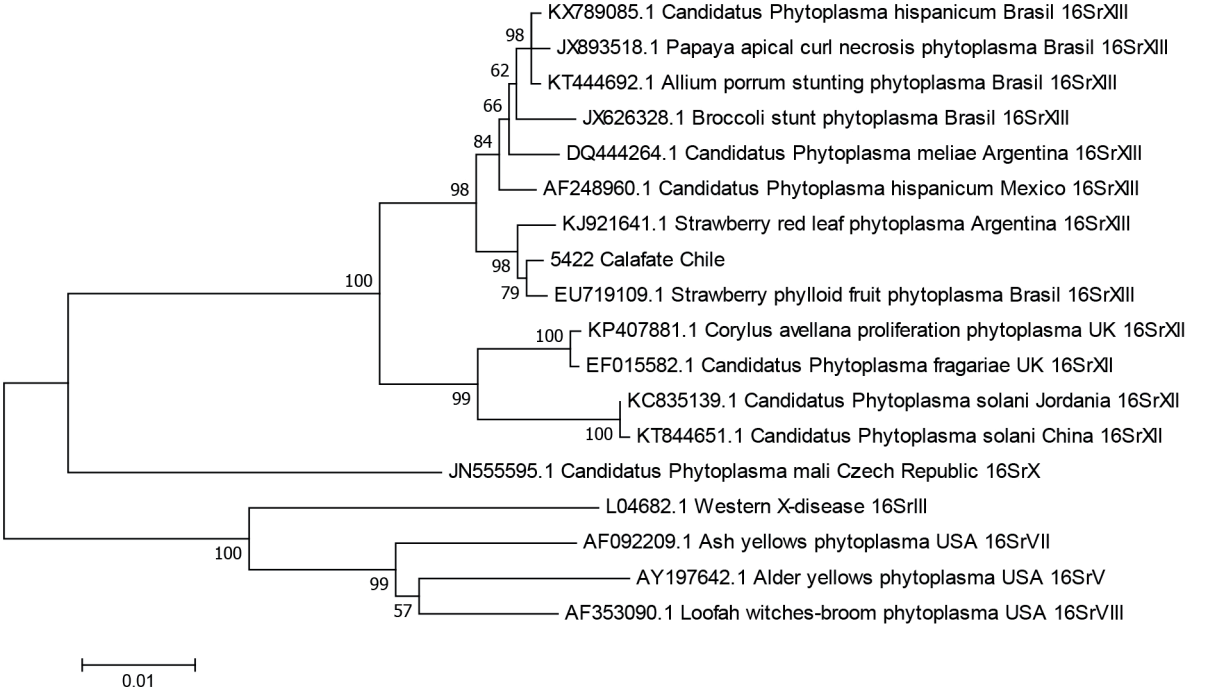
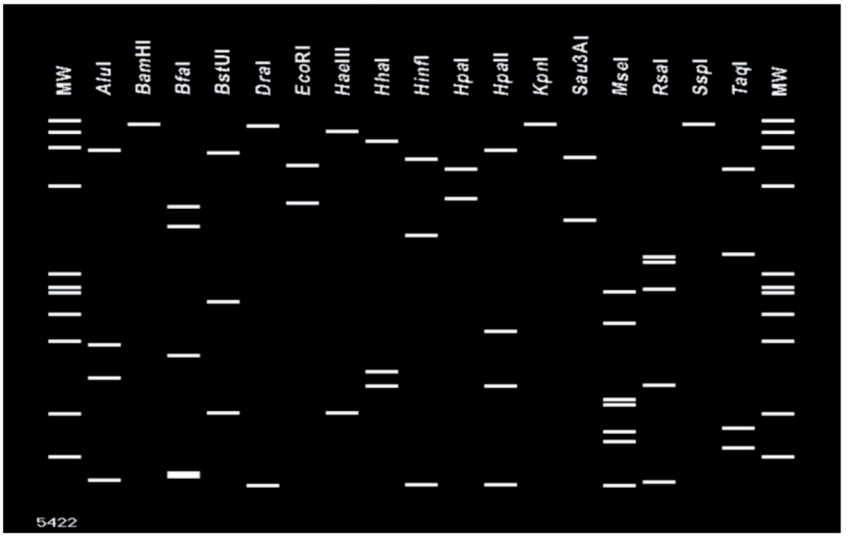


Figure 4. Virtual restriction fragment length polymorphism (RFLP) pattern done with the online tool *i*PhyClassifier. A nucleotide sequence of 16S rDNA was used, which was digested *in silico* in 17 restriction enzymes. This pattern has a similarity coefficient of 1.00 with the reference pattern from the group 16Sr XIII, subgroup F (GenBank accession: KJ921641).



CONCLUSIONS

Amplification of a fragment from the gene 16S, with the analysis of the corresponding sequence, identified phytoplasmas from calafate plants that showed symptoms of witch's broom, coming from *Candidatus* Phytoplasma hispanicum group 16Sr XIII subgroup F.

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