

Molecular identification of 'Candidatus Phytoplasma fraxini' in murta and peony in Chile

Nolberto ARISMENDI¹, Flor GONZÁLEZ², Alan ZAMORANO², Nancy ANDRADE³, Ana Maria PINO², Nicola FIORE²

¹Escuela de Graduados, Facultad de Ciencias Agrarias, University Austral of Chile, Valdivia, Chile

²Departamento de Sanidad Vegetal, Facultad de Ciencias Agronómicas, University of Chile, Santiago, Chile

³Instituto de Producción y Sanidad Vegetal, Facultad de Ciencias Agrarias, University Austral of Chile, Valdivia, Chile

Abstract

Plants of murta (*Ugni molinae* Turcz.) and peony (*Paeonia lactiflora* Pall.), exhibiting disease symptoms suggestive of possible phytoplasma infection were observed in Chile. Leaves were collected from six plants per species (three with and three without symptoms) and main leaf midribs were used for phytoplasma testing. Nested polymerase chain reaction (PCR) amplification allowed the detection of phytoplasmas in samples from plants that showed symptoms, but not in those from the asymptomatic ones. Cloning, sequencing and *in silico* restriction fragment length polymorphism (RFLP) of the 16S rRNA gene allowed identification of the phytoplasmas into ribosomal subgroup 16SrVII-A for both species.

Key words: *Ugni molinae*, peony, nested-PCR, phytoplasmas, *in silico* RFLP, sequencing.

Introduction

Murta (*Ugni molinae* Turcz., family Myrtaceae) is a native bushy plant present mainly in the South of Chile, between the region of Maule (VII) to Aysén del General Carlos Ibáñez del Campo (XI). The berries and leaves are high in antioxidant and analgesic compounds that are used in pharmacology. In murta witches' broom symptoms were observed during spring and summer time. During this period, leaves are smaller and yellowing. At the end of summer and during the autumn season, leaves turn reddish and twigs become necrotic and die. The berries, if present, are smaller and poor in sugar and flavorings. The first report of this disease, based on symptoms, has been made in the early '80s (Novoa, 1982). However, the first laboratory evidence for the presence of phytoplasmas in murta was obtained recently (Andrade *et al.*, 2009).

Peony (*Paeonia lactiflora* Pall., family Paeoniaceae) is cultivated mainly in the regions of Los Ríos (XIV), Araucanía (IX) and Libertador Bernardo O'Higgins (VI) and has been the main species of flowers exported by Chile in 2010, with shipments totaling 1.3 million US dollars. In one orchard of the VI region, plants of the peony variety Henry Bockstoe (*Paeonia lactiflora* x *officinalis*), with deep red flowers, showed: malformation, necrosis and downward rolling of leaves; green stripes on the petals; and drying up of flower bud. No fungal or bacterial isolation was obtained, and no viruses were found in both species. Molecular analyses were carried out during 2010 both in asymptomatic and symptomatic plants to verify the presence of phytoplasmas in murta and peony samples.

Materials and methods

Samples were collected in summer 2010 from three symptomatic and three asymptomatic plants of both species. Total nucleic acids were extracted from 1 g of main leaf

midribs (Prince *et al.*, 1993), dissolved in Tris-EDTA pH 8.0 buffer, and maintained at 4°C; 20 ng/μl of nucleic acid were used for amplification. After direct polymerase chain reaction (PCR) with primer pair P1/P7, nested PCR with R16F2n/R2 primers (Gundersen and Lee, 1996) was performed. PCR and nested PCR reactions were carried out following the published protocol of Schaff *et al.* (1992). Selected R16F2n/R2 amplicons were purified using Concert Rapid PCR Purification System (Life Technologies, Gaithersburg, MD, USA). DNA fragments were ligated into T/A cloning vector pTZ57R/T following the instructions of the InsT/Aclone PCR Product Cloning Kit (Fermentas, MBI, Vilnius, Lithuania). Putative recombinant clones were analysed by colony-PCR, sometimes in combination with the insert-specific primers. Amplicons obtained from three colonies per cloned fragment were sequenced in both directions using the BIG DYE sequencing terminator kit (PE Biosystems, Warrington, UK). The sequences were then aligned with BLAST engine for local alignment (version Blast N 2.2.12). Identification was done using *in silico* restriction fragment length polymorphism (RFLP) analyses on sequences belonging to ribosomal DNA amplified with primer pair R16F2n/R2 with *AluI* and *TaqI* restriction enzymes (Wei *et al.*, 2007).

Results and discussion

Positive results were obtained only after nested amplification on P1/P7. Cloned fragments from R16F2n/R2 amplicons was sequenced and subjected to *in silico* RFLP analysis that placed the phytoplasma in ribosomal subgroups 16SrVII-A ('Candidatus Phytoplasma fraxini') (figure 1). This phytoplasma was detected in all plants with symptoms from both species, but not in the asymptomatic ones. In all cases there was no sequence difference between the three cloned R16F2n/R2 fragments from the same sample (1,240 bp).

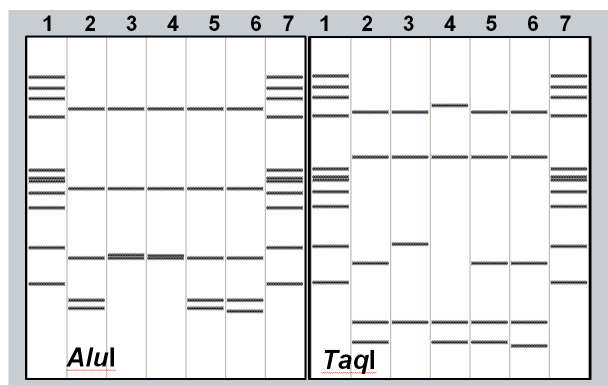


Figure 1. *In silico* RFLP analysis. 1 and 7: PhiX174: marker Φ X174 *Hae*III digested. Phytoplasmas: 2: 16SrVII-A, AF092209; 3: 16SrVII-B, AY034608; 4: 16SrVII-C, AY147038; 5: Chilean murta; 6: Chilean peony.

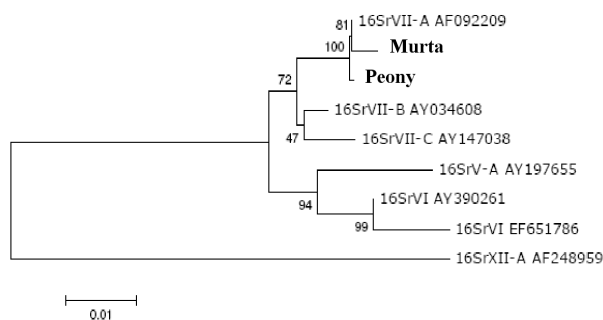


Figure 2. Phenetic tree constructed using neighbor-joining method with 16S rDNA region sequences of two phytoplasm strains from Chilean murta and peony, and related phytoplasmas.

The 16S rDNA (figure 2) sequences from murta and peony were 99.6 and 99.7% identical to AshY1 and AshY5 (Ash yellows phytoplasmas - '*Ca. P. fraxini*' - 16SrVII-A) from the United States (Accession numbers AF092209 and AF105316), respectively (Griffiths *et al.*, 1999).

This is the first report of a 16SrVII-A phytoplasm in murta and peony, even though phytoplasmas belonging to the same ribosomal subgroup were reported to infect grapevines in Chile (Gajardo *et al.*, 2009), ash and lilac in the United States and Canada (Griffiths *et al.*, 1999), ash in Colombia (Franco-Lara *et al.*, 2006), and peach in Italy and Canada (Paltrinieri *et al.*, 2003; Zunnoon-Khan *et al.*, 2010).

The evidence gathered to date suggests that the phytoplasm belonging to ribosomal subgroup 16SrVII-A is the most widespread in the different plant species of Chile. The presence of phytoplasmas in Chilean murta and peony is probably the consequence of both vegetative propagation of infected plants and feeding activity of insect vectors. Moreover, the phytoplasm may overwinter in infected vectors and/or in other perennial plants that serve as reservoirs that spread again the phytoplasm in the following spring.

Acknowledgements

Grateful thanks to Assunta Bertaccini for her helpful suggestions and critical reading of the manuscript. Grant for Doctoral thesis, AT-24100081, CONICYT, Chile.

References

- ANDRADE N., VILLAGRA M., ARISMENDI N., 2009.- Evidencias microscópicas y moleculares de la presencia de fitoplasmas en plantas de murta (*Ugni molinae* Turcz.) afectadas por la enfermedad "escoba de bruja".- *Tropical Plant Pathology* 34: 245-249.
- FRANCO-LARA L., GUTIERREZ C., FILGUEIRA J. J., SALCEDO E., 2006.- Classification of phytoplasmas from *Fraxinus* by using 16S rRNA and tuf genes.- *16th International Congress of the IOM, Cambridge, UK, 9-14 July*, 84: 129.
- GAJARDO A., FIORE N., PRODAN S., PALTRINIERI S., BOTTI S., PINO A. M., ZAMORANO A., MONTEALEGRE J., BERTACCINI A., 2009.- Phytoplasm associated with grapevine yellows disease in Chile.- *Plant Disease*, 93: 789-796.
- GRIFFITHS H. M., SINCLAIR W. A., SMART C. D., DAVIS R. E., 1999.- The phytoplasm associated with ash yellows and lilac witches' broom: '*Candidatus Phytoplasma fraxini*'.- *International Journal of Systematic Bacteriology*, 49: 1605-1614.
- GUNDERSEN D. E., LEE I.-M., 1996.- Ultrasensitive detection of phytoplasm by nested-PCR assays using two universal primer pairs.- *Phytopathologia Mediterranea*, 35: 144-151.
- NOVOA R., 1982.- Antecedentes sobre establecimiento y producción de frutos de la especie *Ugni molinae* Turcz.- Tesis de Ingeniero Forestal, Universidad Austral de Chile, Facultad de Ingeniería Forestal. Valdivia, Chile. 101 pp.
- PALTRINIERI S., DAVINO S., BOTTI S., DAVINO M., BERTACCINI A., 2003.- Identificazione molecolare di fitoplasmi associati ad un deperimento del pesco in Sicilia, pp. 293-296. In: *Atti IV Convegno Nazionale sulla peschicoltura meridionale.- Campobello di Licata* (CARUSO T., MARRA F. P., SOTTILE F., Eds).- AG, 11-12 Settembre 2003.
- PRINCE J. P., DAVIS R. E., WOLF T. K., LEE I.-M., MOGEN B. D., DALLY E. L., BERTACCINI A., CREDI R., BARBA M., 1993.- Molecular detection of diverse mycoplasmalike organisms (MLOs) associated with grapevine yellows MLOs.- *Phytopathology*, 83: 1130-1137.
- SCHAFF D. A., LEE I.-M., DAVIS R. E., 1992.- Sensitive detection and identification of mycoplasmalike organisms by polymerase chain reactions.- *Biochemistry Biophysics Research Communications*, 186: 1503-1509.
- WEI W., DAVIS R. E., LEE I.-M., ZHAO Y., 2007.- Computer-simulated RFLP analysis of 16S rRNA genes: identification of ten new phytoplasm groups.- *International Journal of Systematic and Evolutionary Microbiology*, 57: 1855-1867.
- ZUNNOON-KHAN S., AROCHA-ROSETE Y., SCOTT J., CROSBY W., BERTACCINI A., MICHELUTTI R., 2010.- First report of '*Candidatus Phytoplasma fraxini*' (group 16SrVII phytoplasm) associated with a peach disease in Canada.- *New Disease Reports*, 21: 20.

Corresponding author: Nicola FIORE (nfiore@uchile.cl), University of Chile, Avenida Santa Rosa, 11315 la Pintana, Santiago, Chile.