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Detection and molecular characterization of a phytoplasma affecting *Prunus persica* L. in Jujuy, Argentina

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Abstract Peach (*Prunus persica* L.) plants with symptoms of yellowing, reddening, curling and leaf necrosis, premature defoliation and internode shortening were observed in production fields in Jujuy province (Argentina). A phytoplasma was detected by PCR using the universal primer pairs P1/P7 and R16F2n/R16R2 in all the symptomatic samples analysed. The RFLP profile of PCR products, amplified with R16F2n/R16R2 primers, shows that this phytoplasma, named Argentinean Peach Yellows (ArPY), belongs to subgroup 16Sr III-B. The phylogenetic analysis of the 1244 bp 16S rDNA cloned sequence, grouped the ArPY phytoplasma into the X-disease group with a closer relationship with CFSD, PssWB and ChTDIII phytoplasmas. This is the first report of a phytoplasma infecting peach trees in Argentina.

Keywords Argentina · Peach · Yellowing · PCR-RFLP · 16S rDNA

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Argentina is one of the world's ten largest peach producers. Most of the production comes from Rio Negro, Mendoza and Buenos Aires provinces, and new production areas have been recently incorporated. Among them is Jujuy province, placed in the NOA region (North West Argentina), with nearly 600 ha covered by peach orchards. Jujuy's fruit production has significantly increased during the recent years focused mainly in early and extra-early maturing cultivars which are the first to reach the domestic market (Informe Frutihortícola 2006).

During 2009–2011 peach trees with symptoms of leaf yellowing, reddening, curling necrosis, little leaf, premature defoliation, internode shortening, die back and progressive decline (Fig. 1) were observed in orchards located in temperate valleys of Jujuy. Leaf symptoms appeared in spring with chlorotic or necrotic leaf lesions, resulting in shot hole-like symptoms. Infected scaffold branches produced few smaller low quality and generally lack of embryo fruits with retarded development. Symptoms begin in young shoots of a branch and progressively spread to the entire

Fig. 1 Symptoms caused by Argentinean Peach Yellows phytoplasma in peach trees, **a**: leaf yellowing, reddening, curling and necrosis, **b**: internode shortening and **c**: internode shortening and leaf deformation

canopy. Similar symptoms have been associated worldwide with infections of phytoplasmas such as the western x-disease, peach yellows leaf roll ('*Candidatus* Phytoplasma pyri') (Seemüller and Schneider 2004) and peach rosette in North America (Scott and Zimmerman 2001); '*Ca*. Phytoplasma aurantifolia', '*Ca*. Phytoplasma solani' and '*Ca*. Phytoplasma trifolii' in Iran (Zirak et al. 2010), Peach Yellows in India (Lee et al. 2004); Peach Yellows leaf roll-like disease ('*Ca.* Phytoplasma australiense') in Bolivia (Jones et al. 2005) and the European stone fruit yellows -ESFY- ('*Ca.* Phytoplasma prunorum')

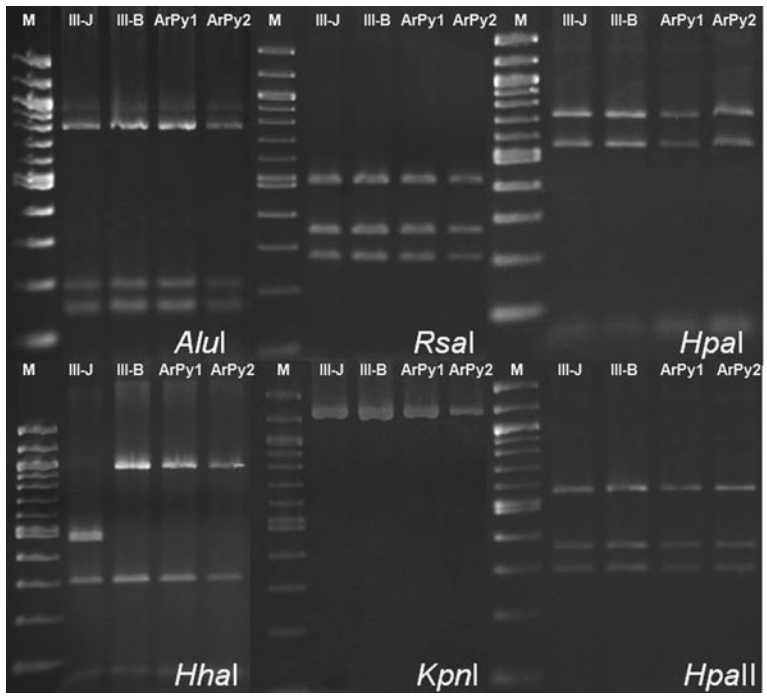

Fig. 2 RFLP profile of R16F2n/R16R2 PCR products from GD: Garlic decline phytoplasma (16Sr III-J); ChTD: China tree decline phytoplasma (16Sr III-B) and ArPy 1, ArPy 2: Argentinean Peach Yellows phytoplasma isolates 1 and 2, with *Alu*I, *RsaI*, *Hha*I, *HpaI*, *HpaII* and *KpnI* restriction enzymes. M: 100pb DNA ladder 100, 200, 300, 400, 500, 600, 700, 800, 900, 1000 and 1500pb (NEB, USA)

in Europe (Seemüller and Schneider 2004). There are no records, however, of phytoplasmas affecting peach trees in Argentina, therefore the aim of the present work was to identify and characterize the etiological agent of this disease that affects peach trees in Northwestern Argentina.

Eight samples of symptomatic and three asymptomatic peach trees *cv.* Flordaking were collected in orchards from Jujuy province during 2009–2011. Total DNA was extracted according to Doyle and Doyle (1990) protocol. The DNA concentration was determined spectrophotometrically and the quality evaluated by agarose gel electrophoresis. PCR reactions were carried out using the universal primer pair P1/P7 (Deng and Hiruki 1991; Schneider et al. 1995) followed by a second round PCR with R16F2n/R16R2 (Gundersen and Lee 1996) as nested primers. Nested PCR products of 1.2 kb were obtained in all the symptomatic peach trees analyzed. No PCR product was observed in asymptomatic samples

In order to identify the phytoplasma, RFLP analysis was performed on the 1.2 kb fragment amplified with R16F2n/R16R2 primers, using endonucleases *Mse*I, *Alu*I, *Rsa*I, *Hha*I, *Hpa*I, *Hpa*II and *Kpn*I. The RFLP profile was compared with those of China tree decline (ChTD) (16SrIII, subgroup B) and Garlic Decline (GD) (16Sr III, subgroup J) (Galdeano et al. 2004) used as references strains for each 16Sr subgroup. The PCR-RFLP profiles of the all tested endonucleases in each phytoplasma-positive tree were identical to each other and indistinguishable from the ChTD (16SrIII, subgroup B). In the Fig. 2 the PCR-RFLP profiles from two representatives ArPY isolates, ChTD and GD are shows. Furthermore, the 1.2 kb PCR product was cloned into pBluescript SKII(+) (Stratagene, USA),

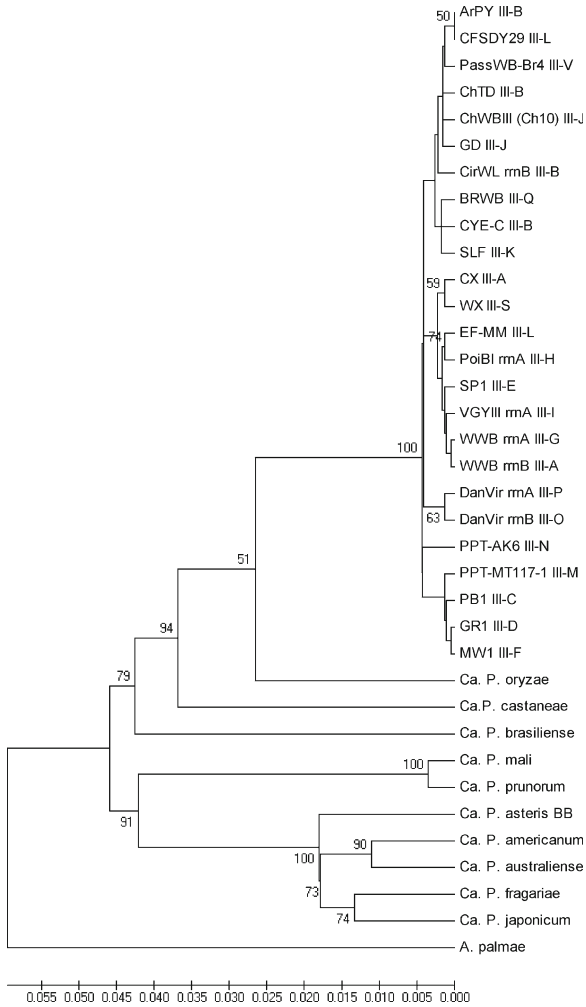

Fig. 3 Phylogenetic tree constructed by UPGMA method of 16S rRNA gene sequences from 24 representative isolates of 16Sr III group, 10 'Ca. Phytoplasmas' and *Acholeplasma palmae* as outgroup. The numbers on the branches are bootstrap (confidence) values. GenBank accessions numbers of phytoplasma 16S rRNA gene sequences: *A. palmae* L33734, Supplementary Material, CirWL (Cirsium white leaf phytoplasma) AF373106, ArPY III-B (Argentinean peach yellows) JQ359014, MW1 III-F (Milkweed yellows phytoplasma) AF510724, PB1 III-C (Pecan bunch phytoplasma) FJ376626, PPT-AK6 III-N (Alaska potato purple top phytoplasma) FJ376629, PPT-MT117 III-M (Montana potato purple top phytoplasma) FJ226074, PassWB-Br4 III-V (Passion fruit witches'-broom phytoplasma) GU292082, CFSDY29 III-L (Cassava frog-skin disease) AY737647, GR1 III-D (Goldenrod yellows phytoplasma) FJ376627, BRWB III-Q (Black raspberry witches'-broom phytoplasma) AF302841, ChTD III-D (China tree decline) AY081817, ChWBIII (Ch10) III-J (Chayote witches'-broom phytoplasma) AF147706, GD III-J (Garlic decline) AY081816, CYE-C III-C (Clover yellow edge phytoplasma) AF175304, SLF III-K (Strawberry leafy fruit phytoplasma) AF274876, CX III-A (Peach X-disease phytoplasma) L33733, WX III-S (Western peach X-disease phytoplasma) L04682, WWB rrnA III-G (Walnut witches'-broom phytoplasma) AF190226, WWB rrnB III-A (Walnut witches'-broom phytoplasma) AF190227, EF-MM III-L (Poinsettia exuberant flower-inducing phytoplasma) EU169138,PoiBI rrnA III-H (Poinsettia branch-inducing phytoplasma) AF190223, SP1 III-E (Spiraea stunt phytoplasma) AF190228, VGYIII rrnA III-I (Virginia grapevine yellows phytoplasma) AF060875, DanVir rrnB III-O (Dandelion virescence phytoplasma) AF370120, DanVir rrnA III-P (Dandelion virescence phytoplasma) (AF370120), Ca. Phytoplasma castaneae AB054986, Ca. Phytoplasma mali AJ542541,Ca. Phytoplasma prunorum AJ542544,Ca. Phytoplasma japonicum AB010425, Ca. Phytoplasma fragariae DQ08642,Ca. Phytoplasma australiense L76865,Ca. Phytoplasma brasiliense AF147708, Ca. Phytoplasma oryzae AB052873, Ca. Phytoplasma asteris BB AY180955 and Ca. Phytoplasma americanum DQ174122

and *E. coli* DH5α strain competent cells were transformed with the recombinant plasmid according to Sambrook et al. (1989). The recombinant clones were sequenced by automated DNA sequencer (Macrogen, Korea). The obtained sequence was recovered after a minimum of 2X sequencing coverage for each base position by overlapping. The sequences were assembled using the SeqMan program (Lasergene software, DNAStar ver. 5, 2001), and manual adjustment was done when necessary. The sequence data was compared with other phytoplasmas 16Sr DNA sequence deposited in GenBank (NCBI). *In silico* analysis of the sequence was carried out using the on line software i*Phy*Classifier (Zhao et al. 2009). Phylogenetic and molecular evolutionary analyses were conducted using MEGA version 5 (Tamura et al. 2011). The 1.2 kb sequence of the ArPY was compared with sequences representative of the X-disease group, other 16Sr groups and *A. palmae* as outgroup.

The ArPY 1244 bp sequence (access number JQ359014) showed 98 % to 99 % identity with phytoplasmas of 16Sr III group. *In silico* RFLP profiles obtained with the *i*PhyClassifier software showed an identical pattern to Clover Yellow Edge phytoplasma, which is the reference strain of subgroup 16Sr III-B. Phylogenetic analysis of 16S rDNA sequences of ArPY, twenty four 16SrIII representative strains and ten '*Ca*. Phytoplasma' species, performed using UPGMA method (1000 bootstrap), grouped ArPY with 16SrIII group phytoplasmas (bott=100) (Fig. 3). In addition, phylogenetic trees constructed by Neighbour-Joining, Maximun Parsimony and Minimun Evolution methods showed a similar topology (data not shown). The ArPY is most closely related to CFSD (Cassava frogskin disease), PssWB (Passion fruit witches'-broom phytoplasma) and ChTDIII (China Tree Decline) phytoplasmas which belong to subgroups 16SrIII-L, -V and -B respectively, all of them found in South America (Galdeano et al. 2004; Alvarez et al. 2009; Davis et al. 2011). This work shows that the symptoms of yellowing, reddening, curling and leaf necrosis, premature defoliation and internode shortening in peach trees were associated with an infection with ArPY phytoplasma, and it is a member of the X-disease group (16SrIII), subgroup B.

This is the first report of a phytoplasma affecting peach trees in Argentina where phytoplasmas of X-disease group have been also detected in China tree (*Melia azeradach*) (Galdeano et al. 2004; Arneodo et al. 2005), garlic (*Allium sativa*) (Galdeano et al. 2004) and several weeds such as *Conyza bonariensis*, *Baccharis flabellata*, *Bidens subalternans var simulans*, *Heterothalamus alienus* and *Caesalpinia gilliesii.* Our results increase the knowledge of phytoplasma diversity present in Argentina and confirm that the X-disease group phytoplasmas are prevalent in the region. In South America, other X-disease group phytoplasmas have been found affecting cassava (*Manihot esculenta*), eggplant (*Solanum melongena*), tomato (*Solanum licopersicum*), coffee (*Coffea arabica*), passion fruit (*Passiflora edulis f. flavicarpa*) and other species (Barros et al. 1998; Amaral Mello et al. 2006; Galvis et al. 2007; Alvarez et al. 2009; Davis et al. 2011). It is interesting to consider the fact that so far almost all X-disease phytoplasmas detected in South America belong to subgroups B and J. The latter was found exclusively in the south area of America. Considering the severe damage caused by the disease on individual plants, it would be necessary to assess the incidence in the production area, estimate the losses that may occur due to the presence of the pathogen and design strategies to minimize the disease impact.

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