Detection and molecular characterization of a 16SrI-F phytoplasma in potato showing ~~a~~ purple top disease in Ecuador

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Abstract

Potato purple top (PPT) disease has been reported worldwide, including Canada, United States, Mexico, Russia and Pakistan. During surveys carried out in a recent epidemic in Ecuador of potato purple top disease phytoplasmas were identified in symptomatic potatoes as well as in some tissue cultured potato shoots by PCR/RFLP analyses and by sequencing and phylogenetic clustering. The majority of the samples resulted positive to phytoplasmas enclosed in the ribosomal subgroup 16SrI-F, for the first time detected in potato and in the American continent. Further studies will allow verification of the transmission and further presence in Ecuador of this phytoplasma associated with a purple top disease of potatoes.

Keywords

Phytoplasmas, Purple top disease, Molecular detection, Sequencing, Potato

Introduction

Potato purple top (PPT) disease has been reported worldwide, including Canada, United States, Mexico, Russia and Pakistan causing significant economic losses (Nasir 2007, Lee et al. 2004, Santos-Cervantes et al. 2010. Several phytoplasmas from 16Sr groups and subgroups have been reported as agents associated with PPT (Lee et al. 2004, Santos-Cervantes et al. 2010, Caicedo et al. 2015).

In 2013, in the northern potato production region of Ecuador, symptoms of ~~potato purple top~~PPT appeared in the fields on scattered plants. Two years later the disease~~d plants resulted~~incidence was ~~to be~~ more than 80% in the Superchola variety fields, with losses reaching up 50% (INIAP~~,~~ 2014, AGROCALIDAD 2015). Characteristic symptomatology of this disease such as yellow and purple coloration of the upper leaflets, apical leaf curling, axillary buds, aerial tubers and early senescence starts to appear after ~~finishing~~ flowering (Fig. 1). In 2015, '*Candidatus* Phytoplasma aurantifolia' (16SrII group) was reported associated with ~~potato purple top disease~~PPT in Ecuador (Caicedo et al. 2015). Considering that the disease was present in several localities, ~~a testing~~surveys were ~~was~~ carried out from asymptomatic and symptomatic potato plants collected in the northern provinces of Carchi

and Pichincha ~~provinces~~ in the highlands in 2015 and 2016 to further confirm phytoplasma presence, identity and association with the disease.

Materials and Methods

~~Plant tissues were frozen at -80°C after collection and tissue selection from different localities and diverse portions of the plants; samples were collected at diverse altitudes and from both s~~Symptomatic (Fig. 1) and asymptomatic potato tissues were~~as~~ collected from different localities and from diverse portions of the plant (Table 1) and stored at -80°C~~s~~. Micropropagated material was also tested ~~(Table 1)~~. DNA was extracted using a CTAB method described by Ferreira and Grattapaglia (1998). The universal phytoplasma primer pair P1/P7 (Deng and Hiruki 1991; Schneider et al. 1995) was used to ~~prime amplification of~~amplify a 1.8 kb fragment of the ribosomal operon consisting of the 16S rRNA gene, the 16S-23S spacer region and a portion of the 5' region of 23S rRNA gene. A 1: 30 dilution of the~~is~~ PCR product was used as template for nested PCR with the primer pair R16(I)F1/R1 which amplifies an internal DNA fragment of 1,100 bp in the 16S rRNA gene (Lee et al. 1994). After the initial denaturation step ~~at~~of 94°C for 1 min, PCR was performed for 35 cycles ~~which were conducted~~ in an automated thermal cycler (Biometra, Germany) ~~each~~ at 94°C for 1 min, 55°C for 2 min (50°C for nested PCR) and 72°C for 3 min, followed by a final extension step at 72°C for 7 min. Phytoplasma positive controls ~~employed for the molecular analyses~~ included DNA from phytoplasma strains maintained in periwinkle [*Catharanthus roseus* (L.) G. Don.] (Bertaccini, 2014): primula yellows from Germany (PRIVA, 16SrI-L); aster yellows from USA (AY, 16SrI-B); *Achillea* yellows from Italy

(ACH, 16SrI-C) and aster yellows from apricot from Spain (A-AY, 16SrI-F). Samples devoid of DNA template were added as negative controls for the direct and nested PCR reactions. PCR mixtures (25 µl) containing 20-60 ng of total DNA, 0.5 µM of each primer, 0.025 U of Taq DNA polymerase (Sigma Aldrich), 1X PCR buffer containing 1.5 mM $MgCl_2$ and 0.2 mM dNTPs. PCR products (6 µl) were electrophoresed ~~through~~ ion ~~o~~n 1% agarose gel, stained with ethidium bromide and visualized with a UV transilluminator.

RFLP analyses of amplified fragments were performed using 100–200 ng DNA per sample that was digested separately with the restriction enzymes *Tru1*I, *Rsa*I and *Taq*I (Fermentas, Vilnius, Lithuania and New England Biolabs) according to the manufacturer's instructions and the ~~amplicon~~ digested amplicon. The restriction products were then separated by electrophoresis through a 6.7% polyacrylamide gel, ~~and~~ stained and visualized as described above. Direct sequencing was carried out in both directions from selected positive samples with primers R16(I)F1/R1 after purification with a QIAquick PCR Purification Kit (QIAGEN, Valencia, CA). The sequences obtained were assembled by MEGA6 (Tamura et al. 2013) ~~and employed~~ for phylogenetic analysis. The evolutionary history was inferred ~~by~~ using the Maximum Likelihood method based on the Tamura-Nei substitution model (Tamura and Nei 1993). Initial tree(s) for the heuristic search were obtained automatically by applying Neighbor-Joining and BioNJ algorithms to a matrix of pairwise distances estimated using the Maximum Composite Likelihood (MCL) approach, and then selecting the topology with superior log likelihood value. Evolutionary analyses were conducted in MEGA6.

Further PCR product analyses were carried out ~~in PCR~~ to verify the presence of '*Candidatus* Liberibacter solanacearum' (Lso) using primers OA2 and OI2c (Liefting et al. 2008~~;~~ 2009) according to the methodology described by Crosslin et al. (2011) and

93 employing the *Taq* DNA polymerase recombinant (Termo Fischer Scientific). PCR
94 products were visualized using agarose gel electrophoresis. Positive controls were obtained
95 from Dr Joseph Munyaneza´s laboratory at USDA-ARS in WA, USA, which showed clear
96 bands corresponding to the expected 1168-bp products (Liefting et al. 2009), with negative
97 controls ~~were~~ as ~~reported~~ described above. Further verification~~s~~ was~~ere~~ performed with the
98 same primers and under the same cycling conditions using 0.16 µl (1.25 units) Promega
99 GoTaq G2 DNA polymerase (Promega) and using primers Lso TX 16/23 F/R under
100 reported conditions which showed the expected 385 bp product in the control samples
101 (Ravindran et al. 2011).
102
103 **Results**
104
105 The direct amplification of ~~PCR for~~ phytoplasma DNA did not result in any
106 detectable positive reaction~~sdetection do not allow to obtain positive results~~ from any of the
107 potato samples, ~~and~~ while only the positive control DNAs ~~from control strains~~ resulted in
108 the expected 1,800 bp amplicons. However, ~~T~~the use of nested PCR with R16(I)F1/R1
109 primers resulted in the expected 1,100 bp amplicons in the majority of the ~~potato~~ samples
110 tested ~~respectively~~ from symptomatic field plants ~~in field or~~and from ~~shoots in~~
111 micropropaged shoot~~ation~~. In some cases the nested PCR results were in contrast to the
112 symptomology. In particular, symptomatic tissue from one aerial tuber, ~~and~~ one stolon ~~from~~
113 ~~symptomatic plants~~ and one micropropagated shoot ~~resulted negative~~gave a negative result
114 while asymptomatic tissue from a sprout, ~~and~~ petiol and one micropropagated shoot

~~resulted positive to phytoplasma presence~~gave a positive result (Table 1). The negative controls were always negative in both direct and nested PCR (data not shown).

RFLP and sequence analysis verified that the Ecuadorian phytoplasmas from potato samples were similar to A-AY control sample from Spain (Lee et al. 1998). The RFLP ~~analyses allow to verify that the~~ profile with restriction enzymes *Tru1*I, *Rsa*I and *Kpn*I was identical to the ~~one of~~ A-AY ~~employed as~~ control (Fig. 2). Likewise, DNA ~~The sequencing~~sequences ~~of three amplicons~~ from samples 7, 12 and 72 ~~allow to obtain 1,025; 1,015 and 1,030 bp respectively that were deposited in~~ (GenBank under accession numbers MG272306, MG272307, MG272308, respectively). ~~The phylogenetic analysis confirmed that the obtained sequences~~ clustered with the A-AY strain (GenBank accession number X68338) (Fig. 3) confirming the RFLP results ~~(Fig. 3)~~. These results sugest that the phytoplasma identified in the potato samples ~~was then classified~~is in the aster yellows cluster, and in ~~particular in~~ subgroup 16SrI-F.

No bands were obtained in the testing for '*Ca*. L. solanacearum' using all above reported primers and conditions from the tested samples, except for the positive controls (data not shown).

Discussion

The e~~E~~pidemiology of ~~purple top~~PPT in Ecuadorian ~~the~~ fields ~~seems to~~ corresponds to a complex of new emergent pathogens such as phytoplasmas located in the plant phloem, spread by infected seed and insect-vectors and mainly detected through molecular analyses~~,~~ ~~such as phytoplasmas~~. Diseases caused by other pathogens like *Rhizoctonia*, which

produces sclerotia on tubers, rot and blight in the stems, and aerial tubers near the base of the plant (main factor that misleads farmers to identify the causal agent of purple top in potato plants in Ecuador), do no present the same ~~behavior~~ symptoms in the field as phytoplasma ~~presence~~. At the high point~~When the high pick~~ of the purple top outbreak ~~started~~ in the Carchi province in March of 2015, symptoms were visualized in more than ~~the~~ 80% of the fields of the potato production area (AGROCALIDAD 2015). The~~is~~ rapid spread of the symptomatic plants does not correspond to the epidemiologi~~e~~y ~~behavior~~ of other pathogens such as *Rhizoctonia*~~,~~. Although, *Rhizoctonia* was, ~~which was~~ in fact, isolated from some PPT symptomatic plants along with the viruses PVY, PVX and PRLV, and *Fusarium cylindrocarpun*, *Pectobacterium* sp. and *Pseudomonas viridiflava* (Castillo et al. 2017), the epidemiological behaviors and the symptoms correspond to infections associated with phytoplasma~~s~~ presence. The ~~epidemiologic behavior observed~~transmission of PPT ~~correspond to~~suggests more similarity to ~~-~~pathogens transmitted by insect vectors and/or spread by infected propagation materials ~~and~~than ~~not~~ to a spreading of a soil fungus like *Rhizoctonia* ~~spreading, for example~~. A previous study detected the presence of '*Candidatus* Phytoplasma aurantifolia' (16SrII group) (Caicedo et al. 2015). ~~and this~~Our study allows to verify the presence of another phytoplasma enclosed in 16SrI-F subgroup. Phytoplasmas enclosed in this subgroup ~~were~~ has only been reported in Spain (strain ACLR-AY = A-AY) and in Germany (strain CVB) (Lee et al. 1998; Bertaccini~~,~~ 2014) in apricot and periwinkle respectively. Our study is the first report of the 16SrI-F subgroup ~~and it is now reported for the first time~~ in potatoes.

~~From literature plants with the same symptomatology resulted~~Past studies have shown that similar symptomatology has resulted in the detection of ~~harbouring~~ both '*Candidatus* Phytoplasma' ~~species~~ and '*Candidatus* Liberibacter solanacearum' in potatoes

(Gutiérrez et al. 2013) and in carrots (Satta et al. 2017). ~~, however in this sampling, the latter was not detected. In countries as México and Spain (Canarias), it has been reported the presence of phytoplasmas together with '*Ca*. L. solanacearum' in potatoes showing purple top~~. ~~Nevertheless the results of the present survey indicate that '*Ca*. L. solanacearum' is not present in Ecuador, yet.~~ Nevertheless the results of the present survey indicate that '*Ca*. L. solanacearum' is not currently present in Ecuador. More research is needed to verify if this phytoplasma is inducing the symptomatology observed in the field although strong association was observed in the present survey. A pathogen complex might be involved in producing purple top symptoms, and therefore further research on other possible prokaryotes together with presence of the insect vector or vectors that ~~might be~~may transmit~~ting~~ these pathogens in the field is necessary, along with a broader sampling in time and space.

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Table 1. Nested PCR detection~~Results~~ of phytoplasma ~~detection~~ in potato ~~samples~~ from Ecuador ~~showing purple top disease using primers R16(I)F1/R1 in nested PCR (in bold samples sequenced)~~.

ID	Plant part	Purple top	Province	Altitude (m asl)	Primer 16SrI
3	Petiol	Symptomatic	Carchi	3070	positive
7	Sprouts[a]	Symptomatic	Carchi	2991	positive
30	Aerial tuber	Symptomatic	Carchi	3070	positive
39	Underground tuber	Symptomatic	Carchi	3070	positive
59	Aerial tuber	Symptomatic	Carchi	3070	positive
60	Aerial tuber	Symptomatic	Carchi	3070	positive
62	Aerial tuber	Symptomatic	Carchi	3070	negative
64	Underground tuber	Symptomatic	Carchi	2991	positive
67	Underground tuber	Symptomatic	Carchi	2991	positive
72	Sprouts[a]	Asymptomatic	Carchi	2991	positive
86	Aereal tuber	Symptomatic	Carchi	3070	positive
12	*In vitro* plant[a]	Asymptomatic	Laboratory	3050	positive
127	*In vitro* plant	Asymptomatic	Laboratory	3050	negative
80	Aereal tuber	Symptomatic	Pichincha	3050	positive
88	Stolon	Symptomatic	Pichincha	3050	negative
103	Flower petiol	Symptomatic	Pichincha	3050	positive
105	Petiol	Symptomatic	Pichincha	3050	positive
114	Petiol	Asymptomatic	Pichincha	2945	positive
117	Petiol	Symptomatic	Pichincha	2945	positive

[a] Sequenced samples

Formattato: Apice

Figure 1. (a) Symptomatic plant in the field (initial, red arrow); (b) curly yellow-purple leaf coloration and (c) aerial tubers located in the upper part of the plant.

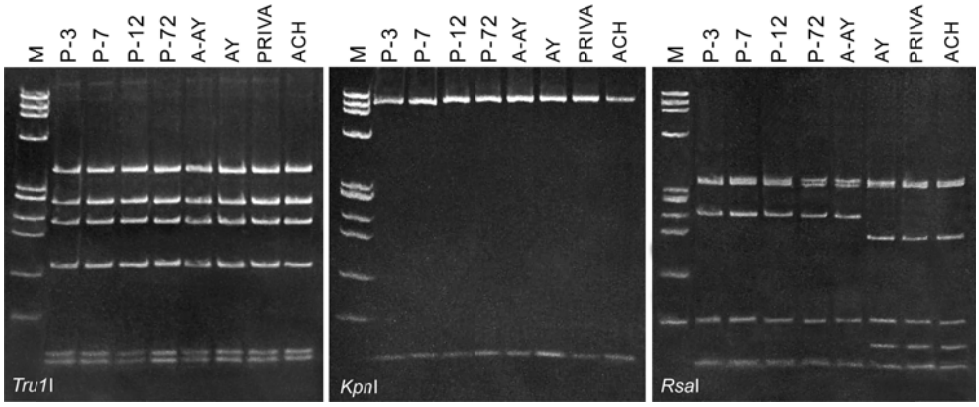

Figure. 2. Polyacrylamide gels 6.7% showing the restriction fragment length polymorphism patterns of R16(I)F1/R1 amplicons from phytoplasma strains from potatoes (P-3, P-7. P12, P72) and controls (A-AY, 16SrI-F; AY, 16SrI-B; PRIVA, 16SrI-L, and ACH, 16SrI-C), the restriction enzymes employed are indicated at the bottom of each gel. M, marker phiX174 DNA digested with *Hae*III length from top to bottom fragments in bp: 1,353; 1,078; 872; 603; 310; 281; 271; 234; 194; 118 and 72.

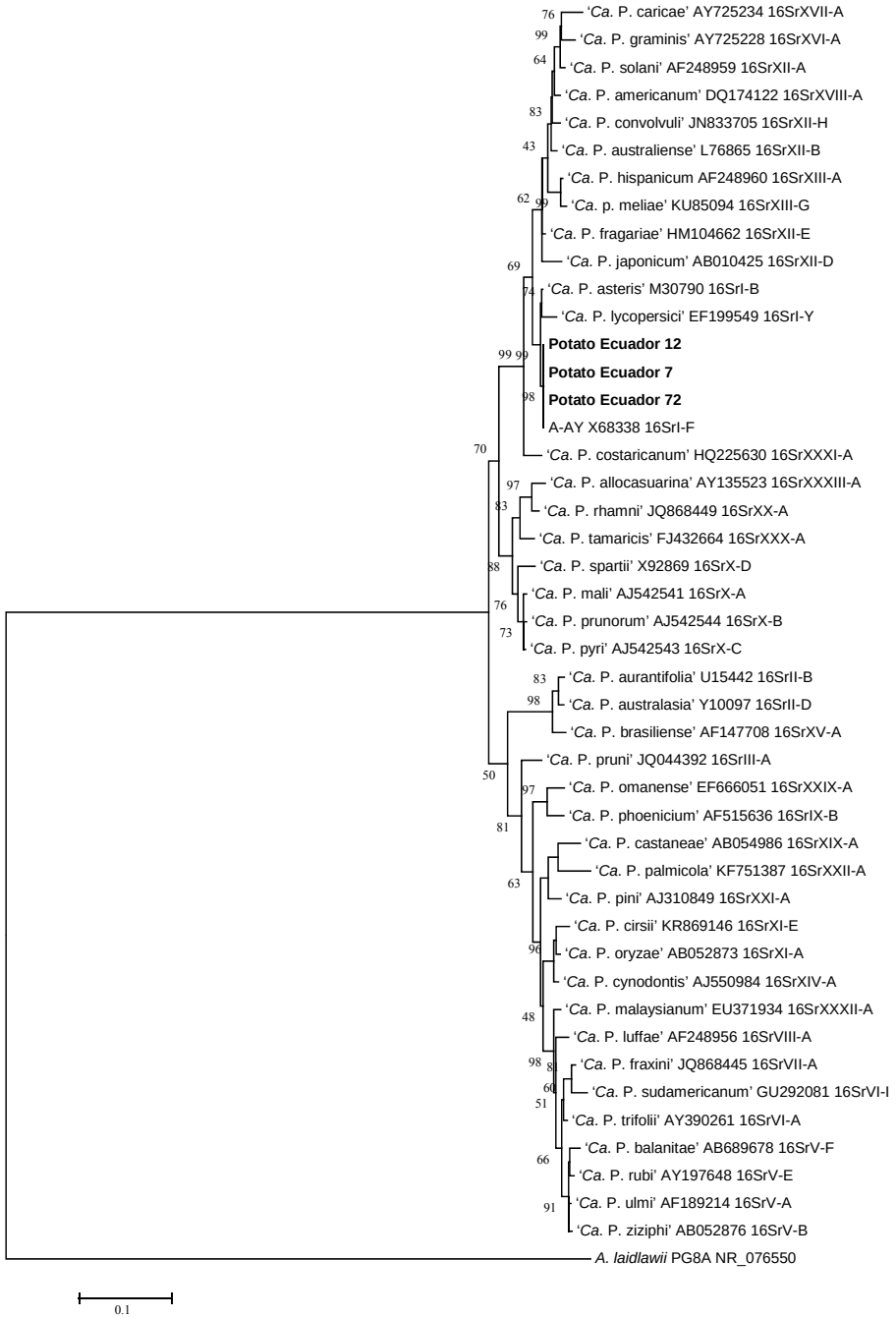
'Ca. P. caricae' AY725234 16SrXVII-A
'Ca. P. graminis' AY725228 16SrXVI-A
'Ca. P. solani' AF248959 16SrXII-A
'Ca. P. americanum' DQ174122 16SrXVIII-A
'Ca. P. convolvuli' JN833705 16SrXII-H
'Ca. P. australiense' L76865 16SrXII-B
'Ca. P. hispanicum AF248960 16SrXIII-A
'Ca. p. meliae' KU85094 16SrXIII-G
'Ca. P. fragariae' HM104662 16SrXII-E
'Ca. P. japonicum' AB010425 16SrXII-D
'Ca. P. asteris' M30790 16SrI-B
'Ca. P. lycopersici' EF199549 16SrI-Y
Potato Ecuador 12
Potato Ecuador 7
Potato Ecuador 72
A-AY X68338 16SrI-F
'Ca. P. costaricanum' HQ225630 16SrXXXI-A
'Ca. P. allocasuarina' AY135523 16SrXXXIII-A
'Ca. P. rhamni' JQ868449 16SrXX-A
'Ca. P. tamaricis' FJ432664 16SrXXX-A
'Ca. P. spartii' X92869 16SrX-D
'Ca. P. mali' AJ542541 16SrX-A
'Ca. P. prunorum' AJ542544 16SrX-B
'Ca. P. pyri' AJ542543 16SrX-C
'Ca. P. aurantifolia' U15442 16SrII-B
'Ca. P. australasia' Y10097 16SrII-D
'Ca. P. brasiliense' AF147708 16SrXV-A
'Ca. P. pruni' JQ044392 16SrIII-A
'Ca. P. omanense' EF666051 16SrXXIX-A
'Ca. P. phoenicium' AF515636 16SrIX-B
'Ca. P. castaneae' AB054986 16SrXIX-A
'Ca. P. palmicola' KF751387 16SrXXII-A
'Ca. P. pini' AJ310849 16SrXXI-A
'Ca. P. cirsii' KR869146 16SrXI-E
'Ca. P. oryzae' AB052873 16SrXI-A
'Ca. P. cynodontis' AJ550984 16SrXIV-A
'Ca. P. malaysianum' EU371934 16SrXXXII-A
'Ca. P. luffae' AF248956 16SrVIII-A
'Ca. P. fraxini' JQ868445 16SrVII-A
'Ca. P. sudamericanum' GU292081 16SrVI-I
'Ca. P. trifolii' AY390261 16SrVI-A
'Ca. P. balanitae' AB689678 16SrV-F
'Ca. P. rubi' AY197648 16SrV-E
'Ca. P. ulmi' AF189214 16SrV-A
'Ca. P. ziziphi' AB052876 16SrV-B
A. laidlawii PG8A NR_076550
0.1

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Figure. 3. Molecular Phylogenetic analysis usingby Maximum Likelihood method. http://purl.org/phylo/treebase/phylows/study/TB2:S21871 The tree with the highest log likelihood (-6080.8085) is shown. Bootstrap The percentage of trees in which the associated taxa clustered together is shown next to the branches, only values above 40 are shown. The tree is drawn to scale, with branch lengths measured in the number of substitutions per site. The analysis involved 46Forty-six closely related nucleotide sequences enclosing from the '*Candidatus* Phytoplasma' species and aster yellows (16SrI)available and some strain in the aster yellows (16SrI) group. The sequences of phytoplasmas from potatoes from Ecuador obtained in this work are in bold. On the right of the branch the GenBank accession number of the strains employed and the ribosomal group/subgroup. *Acholeplasma laidlawii* was used as out group to root the tree.