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(Article begins on next page)

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

3

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13

14 **Abstract**

15

16 Potato purple top (PPT) disease has been reported worldwide, including Canada, United
17 States, Mexico, Russia and Pakistan. During surveys carried out in a recent epidemic in

18 Ecuador of potato purple top disease phytoplasmas were identified in symptomatic potatoes
19 as well as in some tissue cultured potato shoots by PCR/RFLP analyses and by sequencing
20 and phylogenetic clustering. The majority of the samples resulted positive to phytoplasmas
21 enclosed in the ribosomal subgroup 16SrI-F, for the first time detected in potato and in the
22 American continent. Further studies will allow verification of the transmission and further
23 presence in Ecuador of this phytoplasma associated with a purple top disease of potatoes.

24

25 **Keywords**

26

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

28

29 **Introduction**

30

31 | Potato purple top (PPT) disease has been reported worldwide, including Canada,
32 | United States, Mexico, Russia and Pakistan causing significant economic losses (Nasir
33 | 2007, Lee et al. 2004, Santos-Cervantes et al. 2010. Several phytoplasmas from 16Sr
34 | groups and subgroups have been reported as agents associated with PPT (Lee et al. 2004,
35 | Santos-Cervantes et al. 2010, Caicedo et al. 2015).

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

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

48

49 | **Materials and Methods**

50

51 | ~~Plant tissues were frozen at -80°C after collection and tissue selection from different~~
52 | ~~localities and diverse portions of the plants; samples were collected at diverse altitudes and~~
53 | ~~from both s~~Symptomatic (Fig. 1) and asymptomatic potato tissues ~~were~~ collected from
54 | ~~different localities and from diverse portions of the plant (Table 1) and stored at -80°Cs.~~

55 | Micropropagated material was also tested ~~(Table 1)~~. DNA was extracted using a CTAB
56 | method described by Ferreira and Grattapaglia (1998). The universal phytoplasma primer
57 | pair P1/P7 (Deng and Hiruki 1991; Schneider et al. 1995) was used to ~~prime amplification~~
58 | ~~of~~amplify a 1.8 kb fragment of the ribosomal operon consisting of the 16S rRNA gene, the
59 | 16S-23S spacer region and a portion of the 5' region of 23S rRNA gene. A 1: 30 dilution of
60 | ~~the~~is PCR product was used as template for nested PCR with the primer pair R16(I)F1/R1
61 | which amplifies an internal DNA fragment of 1,100 bp in the 16S rRNA gene (Lee et al.
62 | 1994). After the initial denaturation step ~~at~~of 94°C for 1 min, PCR was performed for 35
63 | cycles ~~which were conducted~~ in an automated thermal cycler (Biometra, Germany) ~~each~~ at
64 | 94°C for 1 min, 55°C for 2 min (50°C for nested PCR) and 72°C for 3 min, followed by a
65 | final extension step at 72°C for 7 min. Phytoplasma positive controls ~~employed for the~~
66 | ~~molecular analyses~~ included DNA from phytoplasma strains maintained in periwinkle
67 | [*Catharanthus roseus* (L.) G. Don.] (Bertaccini, 2014): primula yellows from Germany
68 | (PRIVA, 16SrI-L); aster yellows from USA (AY, 16SrI-B); *Achillea* yellows from Italy

69 (ACH, 16SrI-C) and aster yellows from apricot from Spain (A-AY, 16SrI-F). Samples
70 devoid of DNA template were added as negative controls for the direct and nested PCR
71 reactions. PCR mixtures (25 µl) containing 20–60 ng of total DNA, 0.5 µM of each primer,
72 0.025 U of Taq DNA polymerase (Sigma Aldrich), 1X PCR buffer containing 1.5 mM
73 MgCl₂ and 0.2 mM dNTPs. PCR products (6 µl) were electrophoresed ~~through~~ in 1%
74 agarose gel, stained with ethidium bromide and visualized with a UV transilluminator.

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

90 Further PCR product analyses were carried out ~~in PCR~~ to verify the presence of
91 '*Candidatus Liberibacter solanacearum*' (Lso) using primers OA2 and OI2c (Liefing et al.
92 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 verifications ~~wasere~~ 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 reactions~~ ~~detection 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, 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 micropropagated ~~shootation~~. ~~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

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

117 RFLP and sequence analysis verified that the Ecuadorian phytoplasmas from potato
118 samples were similar to A-AY control sample from Spain (Lee et al. 1998). The RFLP
119 ~~analyses allow to verify that the~~ profile with restriction enzymes *TruI*, *RsaI* and *KpnI* was
120 identical to the ~~one of~~ A-AY ~~employed as~~ control (Fig. 2). Likewise, DNA ~~The~~
121 ~~sequencing sequences of three amplicons~~ from samples 7, 12 and 72 ~~allow to obtain 1,025;~~
122 ~~1,015 and 1,030 bp respectively that were deposited in~~ (GenBank under accession numbers
123 MG272306, MG272307, MG272308, respectively). ~~The phylogenetic analysis confirmed~~
124 ~~that the obtained sequences~~ clustered with the A-AY strain (GenBank accession number
125 X68338) (Fig. 3) confirming the RFLP results ~~(Fig. 3)~~. These results suggest that the
126 phytoplasma identified in the potato samples ~~was then classified~~ in the aster yellows
127 cluster, and in ~~particular in~~ subgroup 16SrI-F.

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

131

132 Discussion

133

134 The epidemiology of ~~purple top~~PPT in Ecuadorian ~~the~~ fields ~~seems to correspond~~s
135 to a complex of new emergent pathogens such as phytoplasmas located in the plant phloem,
136 spread by infected seed and insect-vectors and mainly detected through molecular analyses;
137 ~~such as phytoplasmas~~. Diseases caused by other pathogens like *Rhizoctonia*, which

138 produces sclerotia on tubers, rot and blight in the stems, and aerial tubers near the base of
139 the plant (main factor that misleads farmers to identify the causal agent of purple top in
140 potato plants in Ecuador), do not present the same ~~behavior-symptoms~~ in the field as
141 phytoplasma ~~presence~~. ~~At the high point~~~~When the high pick~~ of the purple top outbreak
142 ~~started in the~~ Carchi province in March of 2015, symptoms were visualized in more than
143 ~~the~~ 80% of the fields of the potato production area (AGROCALIDAD 2015). ~~The~~ rapid
144 spread of the symptomatic plants does not correspond to ~~the~~ epidemiologic ~~behavior~~ of
145 ~~other~~ pathogens such as *Rhizoctonia*. ~~Although, Rhizoctonia was, which was~~ in fact,
146 isolated from some ~~PPT symptomatic~~ plants along with the viruses PVY, PVX and PRLV,
147 and *Fusarium cylindrocarpum*, *Pectobacterium* sp. and *Pseudomonas viridiflava* (Castillo et
148 al. 2017). ~~the epidemiological behaviors and the symptoms correspond to infections~~
149 ~~associated with phytoplasmas presence~~. The ~~epidemiologic behavior observed~~ ~~transmission~~
150 ~~of PPT correspond to~~ ~~suggests more~~ ~~similarity to~~ -pathogens transmitted by insect vectors
151 and/or spread by infected propagation materials ~~and than not~~ to a ~~spreading of a soil fungus~~
152 ~~like Rhizoctonia spreading, for example~~. A previous study detected the presence of
153 ‘*Candidatus* Phytoplasma aurantifolia’ (16SrII group) (Caicedo et al. 2015). ~~and this~~ ~~Our~~
154 study allows to verify the presence of another phytoplasma enclosed in 16SrI-F subgroup.
155 Phytoplasmas enclosed in this subgroup ~~were has~~ only ~~been~~ reported in Spain (strain
156 ACLR-AY = A-AY) and in Germany (strain CVB) (Lee et al. 1998; Bertaccini, 2014) in
157 apricot and periwinkle respectively. ~~Our study is the first report of the 16SrI-F subgroup~~
158 ~~and it is now reported for the first time~~ in potatoes.

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

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

174

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176

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181

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246 Table 1. Nested PCR detection Results of phytoplasma ~~detection~~ in potato ~~samples~~ from
 247 Ecuador ~~showing purple top disease using primers R16(I)F1/R1 in nested PCR (in bold~~
 248 ~~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	<i>In vitro</i> plant ^a	Asymptomatic	Laboratory	3050	positive
127	<i>In vitro</i> 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

249 ^a Sequenced samples
 250

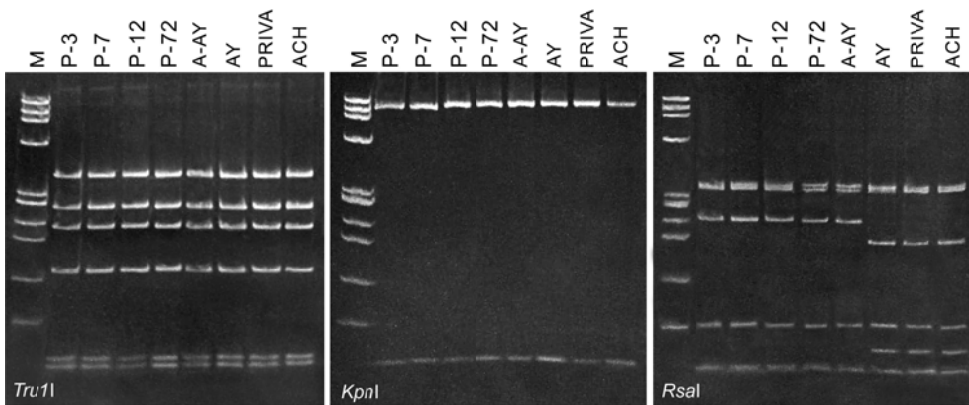
Formattato: Apice



252

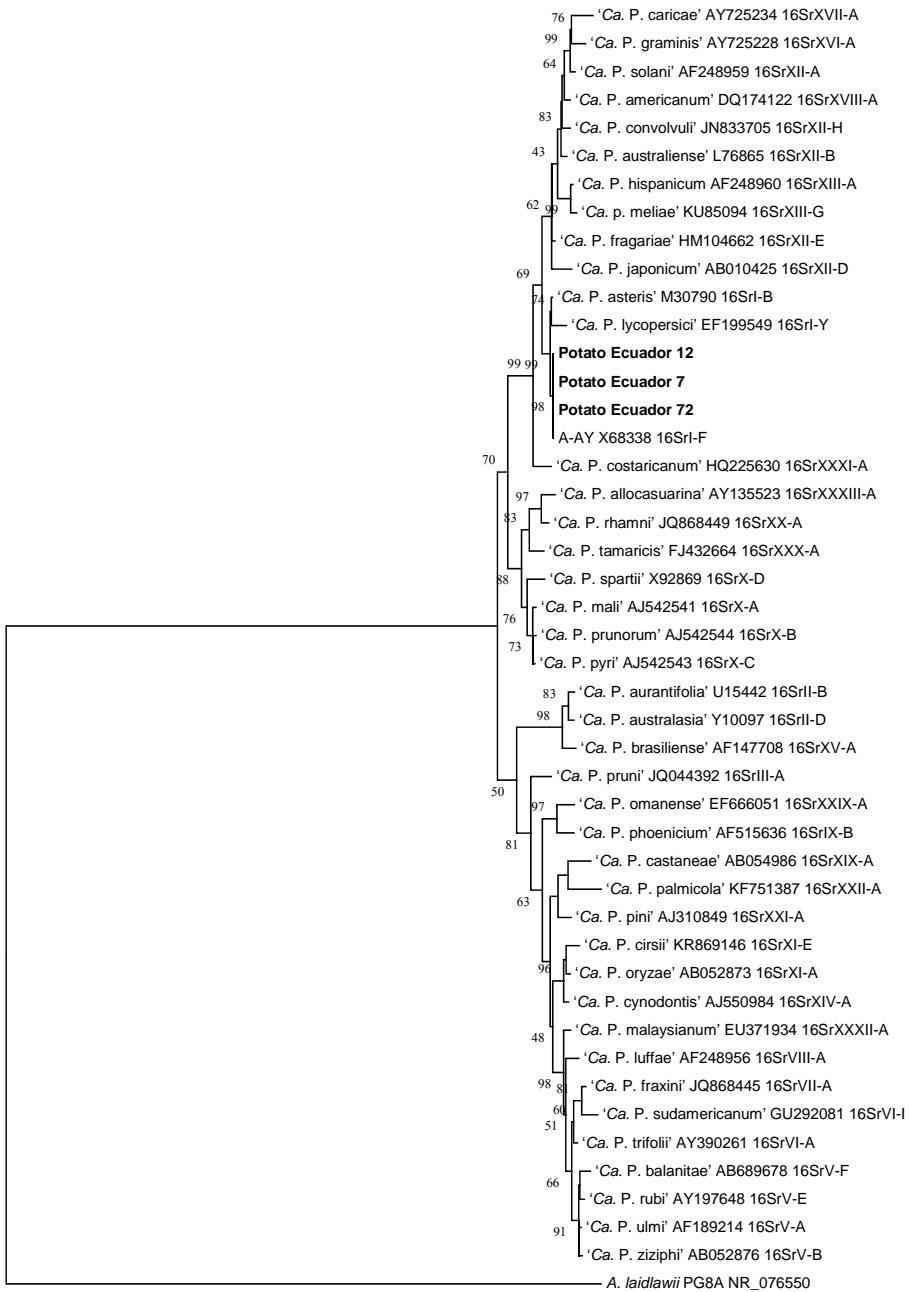
254 Figure 1. (a) Symptomatic plant in the field (initial, red arrow); (b) curly yellow-purple leaf
 255 coloration and (c) aerial tubers located in the upper part of the plant.

255



256

262 Figure 2. Polyacrylamide gels 6.7% showing the restriction fragment length polymorphism
 263 patterns of R16(I)F1/R1 amplicons from phytoplasma strains from potatoes (P-3, P-7, P12,
 264 P72) and controls (A-AY, 16SrI-F; AY, 16SrI-B; PRIVA, 16SrI-L, and ACH, 16SrI-C), the
 265 restriction enzymes employed are indicated at the bottom of each gel. M, marker phiX174
 266 DNA digested with *Hae*III length from top to bottom fragments in bp: 1,353; 1,078; 872;
 267 603; 310; 281; 271; 234; 194; 118 and 72.



263 | Figure. 3. Molecular Phylogenetic analysis ~~using~~ Maximum Likelihood method.
264 | <http://purl.org/phylo/treebase/phyloids/study/TB2:S21871> The tree with the highest log
265 | likelihood (-6080.8085) is shown. ~~Bootstrap. The percentage of trees in which the~~
266 | ~~associated taxa clustered together is shown next to the branches, only~~ values above 40 are
267 | shown. ~~The tree is drawn to scale, with branch lengths measured in the number of~~
268 | ~~substitutions per site. The analysis involved 46~~Forty-six closely related nucleotide
269 | sequences ~~encloding from~~ the ‘*Candidatus* Phytoplasma’ species ~~and aster yellows~~
270 | ~~(16SrI)available and some strain in the aster yellows (16SrI) group.~~ The sequences of
271 | phytoplasmas from potatoes from Ecuador obtained in this work are in bold. On the right of
272 | the branch the GenBank accession number of the strains employed and the ribosomal
273 | group/subgroup. *Acholeplasma laidlawii* was used as out group to root the tree.