ARCHIVIO ISTITUZIONALE
DELLA RICERCA

Alma Mater Studiorum Università di Bologna
Archivio istituzionale della ricerca

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

This is the final peer-reviewed author's accepted manuscript (postprint) of the following publication:

Published Version:
Castillo Carrillo, C., Paltrinieri, S., Bustamante, J.B., Bertaccini, A. (2018). Detection and molecular characterization of a 16SrI-F phytoplasma in potato showing purple top disease in Ecuador. AUSTRALASIAN PLANT PATHOLOGY, 47(3), 311-315 [10.1007/s13313-018-0557-9].

Availability:
This version is available at: https://hdl.handle.net/11585/660320 since: 2024-07-30

Published:

DOI: http://doi.org/10.1007/s13313-018-0557-9

Terms of use:

Some rights reserved. The terms and conditions for the reuse of this version of the manuscript are specified in the publishing policy. For all terms of use and more information see the publisher's website.

This item was downloaded from IRIS Università di Bologna (https://cris.unibo.it/).
When citing, please refer to the published version.

(Article begins on next page)

26 December 2024

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

Samanta Paltrinieri[1], Johanna Buitrón Bustamante[2], Assunta Bertaccini[1], Carmen Castillo Carrillo[2]

[1]*Alma Mater Studiorum*– University of Bologna, DipSA, Plant Pathology, viale G. Fanin, 42, 40127 Bologna, Italy

[2]Estación Experimental Santa Catalina, Instituto Nacional de Investigaciones Agropecuarias (INIAP), Departamento Nacional de Biotecnología y Departamento Nacional de Protección Vegetal. Panamericana Sur km 1, Quito, Ecuador

Corresponding author: Carmen Castillo Carrillo, email address: carmen.castillo@iniap.gob.ec

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 PPT appeared in the fields on scattered plants. Two years later the disease was more than 80% in the Superchola variety fields, with losses reaching up 50% (INIAP 2014, E. Reina, personal communication).

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 flowering (Fig. 1). In 2015, '*Candidatus* Phytoplasma aurantifolia' (16SrII group) was reported associated with PPT in Ecuador (Caicedo et al. 2015). Considering that the disease was present in several localities, surveys were carried out from asymptomatic and symptomatic potato plants collected in the northern provinces of

47 Carchi and Pichincha-in the highlands in 2015 and 2016 to further confirm phytoplasma

48 presence, identity and association with the disease.

48

49 **Materials and Methods**

50

51 Symptomatic (Fig. 1) and asymptomatic potato tissues were-collected from

52 different localities and from diverse portions of the plant (Table 1) and stored at -80ºCs.

53 Micropropagated material was also tested–DNA was extracted using a CTAB

54 method described by Ferreira and Grattapaglia (1998). The universal phytoplasma primer

55 pair P1/P7 (Deng and Hiruki 1991; Schneider et al. 1995) was used to amplify a 1.8 kb

56 fragment of the ribosomal operon consisting of the 16S rRNA gene, the

57 16S-23S spacer region and a portion of the 5' region of 23S rRNA gene. A 1: 30 dilution of

58 the PCR product was used as template for nested PCR with the primer pair R16(I)F1/R1

59 which amplifies an internal DNA fragment of 1,100 bp in the 16S rRNA gene (Lee et al.

60 1994). After the initial denaturation step of 94°C for 1 min, PCR was performed for 35

61 cycles in an automated thermal cycler (Biometra, Germany) at

62 94°C for 1 min, 55°C for 2 min (50°C for nested PCR) and 72°C for 3 min, followed by a

63 final extension step at 72°C for 7 min. Phytoplasma positive controls

64 included DNA from phytoplasma strains maintained in periwinkle

65 [*Catharanthus roseus* (L.) G. Don.] (Bertaccini, 2014): primula yellows from Germany

66 (PRIVA, 16SrI-L); aster yellows from USA (AY, 16SrI-B); *Achillea* yellows from Italy

67 (ACH, 16SrI-C) and aster yellows from apricot from Spain (A-AY, 16SrI-F). Samples
68 devoid of DNA template were added as negative controls for the direct and nested PCR
69 reactions. PCR mixtures (25 µl) containing 20-60 ng of total DNA, 0.5 µM of each primer,
70 0.025 U of Taq DNA polymerase (Sigma Aldrich), 1X PCR buffer containing 1.5 mM
71 $MgCl_2$ and 0.2 mM dNTPs. PCR products (6 µl) were electrophoresed in 1%
72 agarose gel, stained with ethidium bromide and visualized with a UV transilluminator.

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

88 Further PCR product analyses were carried out to verify the presence of
89 '*Candidatus* Liberibacter solanacearum' (Lso) using primers OA2 and OI2c (Liefting et al.
90 2008,; 2009) according to the methodology described by Crosslin et al. (2011) and

91 employing the *Taq* DNA polymerase recombinant (Termo Fischer Scientific). PCR
92 products were visualized using agarose gel electrophoresis. Positive controls were obtained
93 from Dr Joseph Munyaneza´s laboratory at USDA-ARS in WA, USA, which showed clear
94 bands corresponding to the expected 1168-bp products (Liefting et al. 2009), with negative
95 controls as described above. Further verification was performed with the
96 same primers and under the same cycling conditions using 0.16 µl (1.25 units) Promega
97 GoTaq G2 DNA polymerase (Promega) and using primers Lso TX 16/23 F/R under
98 reported conditions which showed the expected 385 bp product in the control samples
99 (Ravindran et al. 2011).

102

103 Results

104

105 The direct amplification of phytoplasma DNA did not result in any
106 detectable positive reaction from any of the
107 potato samples, while only the positive control DNAs 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 samples
110 tested from symptomatic field plants and from
111 micropropaged shoot. In some cases the nested PCR results were in contrast to the
112 symptomology. In particular, symptomatic tissue from one aerial tuber, one stolon
113 and one micropropagated shoot gave a negative result
114 while asymptomatic tissue from a sprout, petiol and one micropropagated shoot

115 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 profile with restriction enzymes *Tru1*I, *Rsa*I and *Kpn*I was
120 identical to the A-AY control (Fig. 2). Likewise, DNA
121 sequences-from samples 7, 12 and 72 (GenBank under accession numbers
122 MG272306, MG272307, MG272308, respectively).
123 clustered with the A-AY strain (GenBank accession number
124 X68338) (Fig. 3) confirming the RFLP results. These results sugest that the
125 phytoplasma identified in the potato samples in the aster yellows
126 cluster, and in subgroup 16SrI-F.
127 No bands were obtained in the testing for '*Ca*. L. solanacearum' using all above
128 reported primers and conditions from the tested samples, except for the positive controls
129 (data not shown).
131
132 **Discussion**
133
134 The epidemiology of PPT in Ecuadorian fields corresponds
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 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 no present the same symptoms in the field as
141 phytoplasma. At the high point of the purple top outbreak
142 in Carchi province in March of 2015, symptoms were visualized in more than
143 80% of the fields of the potato production area (AGROCALIDAD 2015). The rapid
144 spread of the symptomatic plants does not correspond to the epidemiology of
145 other pathogens such as *Rhizoctonia*. Although, *Rhizoctonia* was, in fact,
146 isolated from some PPT symptomatic plants along with the viruses PVY, PVX and PRLV,
147 and *Fusarium cylindrocarpun*, *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 transmission
150 of PPT suggests more similarity to pathogens transmitted by insect vectors
151 and/or spread by infected propagation materials than to a spreading of a soil fungus
152 like *Rhizoctonia*. A previous study detected the presence of
153 '*Candidatus* Phytoplasma aurantifolia' (16SrII group) (Caicedo et al. 2015). Our
154 study allows to verify the presence of another phytoplasma enclosed in 16SrI-F subgroup.
155 Phytoplasmas enclosed in this subgroup 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 in potatoes.
159 Past studies have
160 shown that similar symptomatology has resulted in the detection of both
161 '*Candidatus* Phytoplasma' and '*Candidatus* Liberibacter solanacearum' in potatoes

(Gutiérrez et al. 2013) and in carrots (Satta et al. 2017). ,

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 may transmit these pathogens in the field is necessary, along with a broader sampling in time and space.

Acknowledgments

This research was authorized by the agreement between the National Institute of Agronomic Research and the Ministry of Environment of Ecuador reference number MAE-DNB-CM-2015-0024, clause 9.2.5. We thank Dr. Charles W. Barnes, Dr. José Ochoa and the anonymous reviewers for helpful comments and suggestions on the manuscript.

References

184 AGROCALIDAD (2015) Agencia Ecuatoriana de Aseguramiento de la Calidad del Agro.
185 Informe técnico sobre punta morada en papa. Coordinación General de Sanidad Vegetal.
186 Elaborado el 30 de octubre del 2015
187 Bertaccini A (2014) Phytoplasma Collection. International Phytoplasmologists Working
188 Group web. http://www.ipwgnet.org/collection. Accessed 30 October 2017
189 Caicedo J, Crizón M, Pozo A, Cevallos A, Simbaña L, Rivera L, Arahana V (2015) First
190 report of '*Candidatus* Phytoplasma aurantifolia' (16SrII) associated with potato purple top
191 in San Gabriel-Carchi, Ecuador. New Dis Reprt 32
192 Castillo Carrillo C, Buitrón J, Insuasti M, Castillo N, Rivadeneira J, Cuesta X (2017)
193 Avances en el diagnostic del agente causal del problema de punta morada en papa en
194 Ecuador. Memorias del VII Congreso Ecuatoriano de la Papa. Tulcán, Ecuador
195 Crosslin JM, Lin H, Munyaneza JE (2011) Detection of '*Candidatus* Liberibacter
196 solanacearum' in the potato psyllid, *Bactericera cockerelli* (Sulc), by conventional and
197 real-time PCR. Southwest Entomol 36:125–135
198 Deng SJ, Hiruki C (1991) Amplification of 16S ribosomal-RNA genes from culturable and
199 nonculturable mollicutes. J Microbiol Meth 14:53–61
200 Ferreira ME, Grattapaglia D (1998) Introducción al uso de marcadores moleculares en el
201 análisis genético. EMBRAPA (Empresa Brasileira de Pesquisa Agropecuária) -
202 CENARGEN (Centro Nacional de Pesquisa de Recursos Genéticos e Biotecnologia
203 Gutiérrez-Ibáñez AT, Sánchez-Pale JR, Cerda AL, Ramírez DJF, Balbuena AM, Alvarado
204 MA (2013) Detección de '*Ca.* Liberibacter solanacearum' y fitoplasmas en cultivo de papa
205 (*Solanum tuberosum* L.) en el Valle de Toluca. Rev Col de Biotec 15:145–149

INIAP (2014) Informe Técnico Anual. Fitoplasmas asociados a la punta morada de la papa en Ecuador. Instituto Nacional de Investigaciones Agropecuarias, Programa Nacional de Raíces y Tubérculos. Quito, Ecuador. ~~81 pp~~

Lee I-M, Gundersen-Rindal DE, Hammond RD, Davis RE (1994) Use of mycoplasma-like organism (MLOs) group specific oligonucleotide primers for nested-PCR assays to detect mixed-MLO infections in a single host plant. Phytopathol~~ogy~~ 84:559-566

Lee I-M, Gundersen-Rindal DE, Davis RE, Bartoszyk IM (1998) Revised classification scheme of phytoplasmas based on RFLP analyses of 16s rRNA and ribosomal protein gene sequences. Int J Syst Evol Microbiol 48:1153-1169

Lee I-M, Bottner KD, Munyaneza JE, Secor GA, Gudmestad NC (2004) Clover proliferation group (16SrVI) subgroup A (16SrVI-A) phytoplasma is a probable causal agent of potato purple top disease in Washington and Oregon. Plant Dis. 88:429

Liefting LW, Perez-Egusquiza ZC, Clover GRG, Anderson JAD (2008) A new '*Candidatus* Liberibacter' species in *Solanum tuberosum* in New Zealand. Plant Dis 92:1474

Liefting LW, Sutherland PW, Ward LI, Paice KL, Weir BS, Clover GRG (2009) A new '*Candidatus* Liberibacter' species associated with diseases of solanaceous crops. Plant Dis 93:208–214

Nasir MM, Mughal SM, Khan SM (2007) Occurrence, distribution and detection of potato purple top phytoplasma disease in the Punjab (Pakistan). Bull Insectol 60:377-378

Ravindran A, Levy J, Pierson E, Gross DC (2011) Development of primers for improved PCR detection of the potato zebra chip pathogen, '*Candidatus* Liberibacter solanacearum'. Plant Dis 95:1542–1546

Santos-Cervantes ME, Chávez-Medina JA, Acosta-Pardini J, Flores-Zamora GL, Mendez-Lozano J, Leyva-Lopez NE (2010) Genetic diversity and geographical distribution of phytoplasmas associated with potato purple top disease in Mexico. Plant Dis 94:388-395

Satta E, Ramirez AS, Paltrinieri S, Contaldo N, Benito P, Poveda JB, Bertaccini A 2017 Simultaneous detection of mixed '*Candidatus* Phytoplasma asteris' and '*Ca*. Liberibacter solanacearum'infection in carrot. Phytopath Medit 55~~(3)~~:401-409

Schneider B, Seemüller E, Smart CD, Kirkpatrick BC (1995) Phylogenetic classification of plant pathogenic mycoplasma-like organisms or phytoplasmas. In: Razin S, and Tully JG (eds) Molecular and diagnostic procedures in mycoplasmology Academic Press, San Diego

Tamura K, Nei M (1993) Estimation of the number of nucleotide substitutions in the control region of mitochondrial DNA in humans and chimpanzees. Mol Biol and Evol 10:512-526

Tamura K, Stecher G, Peterson D, Filipski A, Kumar S (2013) MEGA6: Molecular Evolutionary Genetics Analysis version 6.0. Mol Biol and Evol 30:2725-2729

244 Table 1. Nested PCR detection of phytoplasma in potato from

245 Ecuador.

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

246 [a] Sequenced samples

250

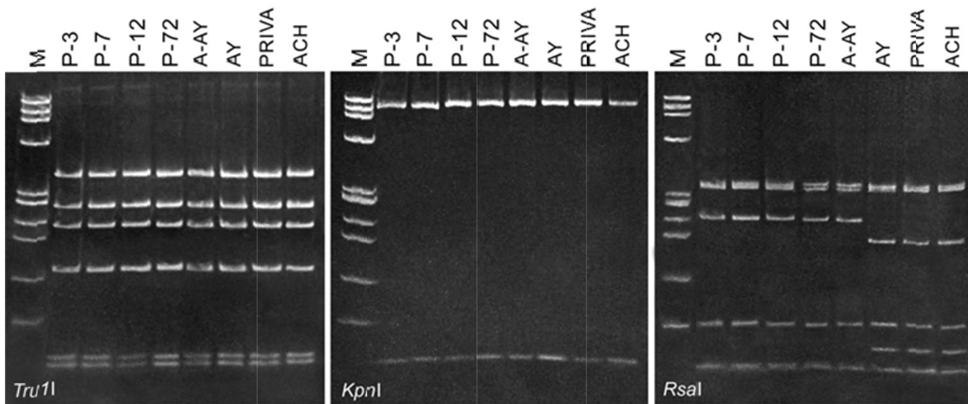
252

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

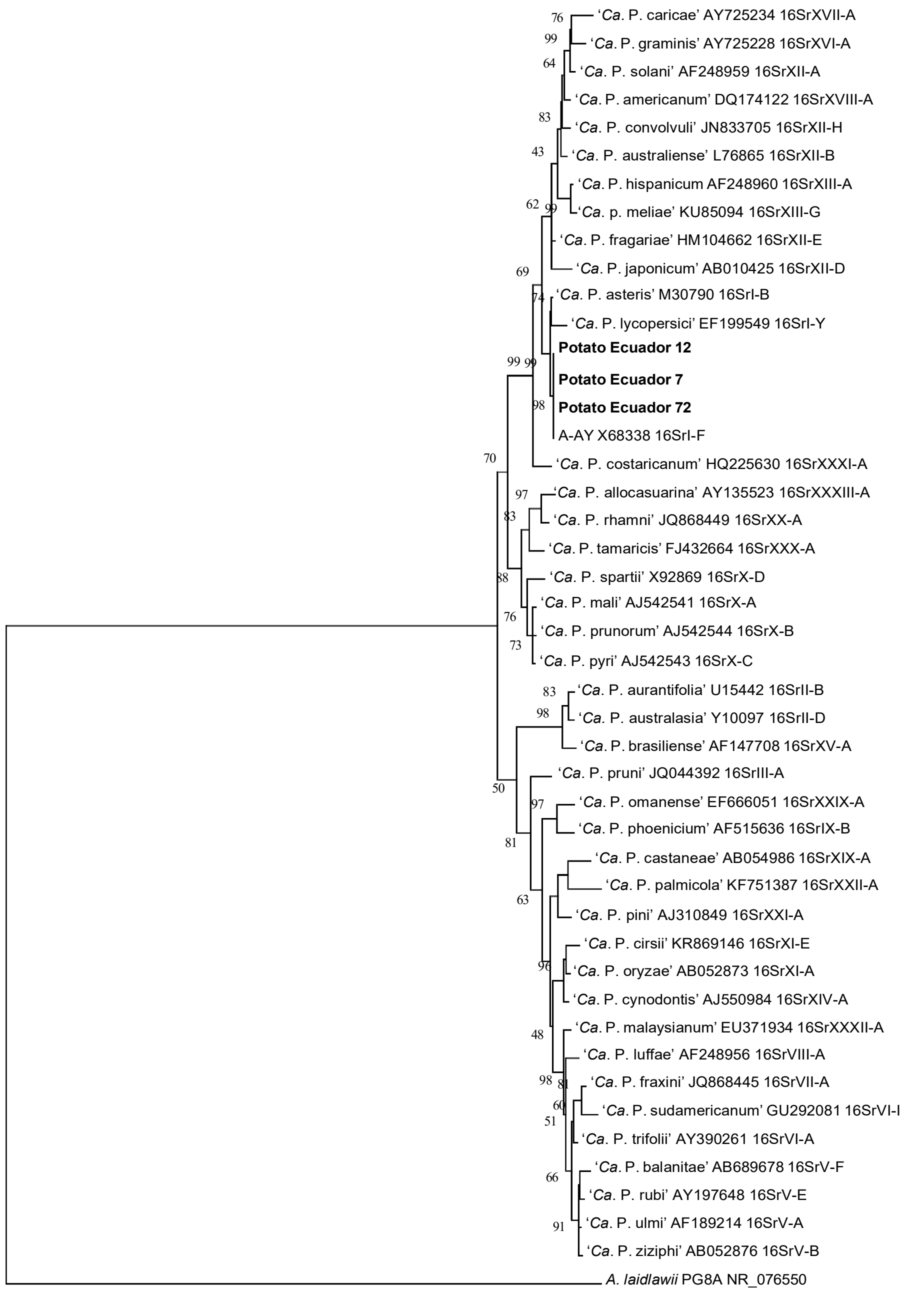
263 patterns of R16(I)F1/R1 amplicons from phytoplasma strains from potatoes (P-3, P-7. P12,

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.



262

263 Figure. 3. Molecular Phylogenetic analysis using Maximum Likelihood method.
264 http://purl.org/phylo/treebase/phylows/study/TB2:S21871 The tree with the highest log
265 likelihood (-6080.8085) is shown. Bootstrap values above 40 are
266 shown. Forty-six closely related nucleotide
267 sequences from the '*Candidatus* Phytoplasma' species and aster yellows
268 (16Srl) The sequences of phytoplasmas from potatoes from Ecuador obtained
269 in this work are in bold. On the right of the branch the GenBank accession
270 number of the strains employed and the ribosomal
271 group/subgroup. *Acholeplasma laidlawii* was used as out group to root the tree.