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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 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 ~~P~~PPT in Ecuador (Caicedo et al. 2015). Considering that the disease was present in several localities, ~~s~~surveys were carried out from asymptomatic and symptomatic potato plants collected in the northern provinces of

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

Materials and Methods

Symptomatic (Fig. 1) and asymptomatic potato tissues were collected from different localities and from diverse portions of the plant (Table 1) and stored at -80ºCs. Micropropagated material was also tested. 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 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 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 of 94°C for 1 min, PCR was performed for 35 cycles in an automated thermal cycler (Biometra, Germany) 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 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 in 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 digested amplicon. The restriction products were then separated by electrophoresis through a 6.7% polyacrylamide gel 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) for phylogenetic analysis. The evolutionary history was inferred 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 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

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

Results

The direct amplification of phytoplasma DNA did not result in any detectable positive reaction from any of the potato samples, while only the positive control DNAs resulted in the expected 1,800 bp amplicons. However, the use of nested PCR with R16(I)F1/R1 primers resulted in the expected 1,100 bp amplicons in the majority of the samples tested from symptomatic field plants and from micropropaged shoot. In some cases the nested PCR results were in contrast to the symptomology. In particular, symptomatic tissue from one aerial tuber, one stolon and one micropropagated shoot gave a negative result while asymptomatic tissue from a sprout, petiol and one micropropagated shoot

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 profile with restriction enzymes *Tru1*I, *Rsa*I and *Kpn*I was identical to the A-AY control (Fig. 2). Likewise, DNA sequences from samples 7, 12 and 72 (GenBank under accession numbers MG272306, MG272307, MG272308, respectively). clustered with the A-AY strain (GenBank accession number X68338) (Fig. 3) confirming the RFLP results. These results sugest that the phytoplasma identified in the potato samples in the aster yellows cluster, and 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 epidemiology of PPT in Ecuadorian fields 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. 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 symptoms in the field as phytoplasma. At the high point of the purple top outbreak in Carchi province in March of 2015, symptoms were visualized in more than 80% of the fields of the potato production area (AGROCALIDAD 2015). The rapid spread of the symptomatic plants does not correspond to the epidemiology of other pathogens such as *Rhizoctonia*. Although, *Rhizoctonia* 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 phytoplasmas presence. The transmission of PPT suggests more similarity to pathogens transmitted by insect vectors and/or spread by infected propagation materials than to a spreading of a soil fungus like *Rhizoctonia*. A previous study detected the presence of '*Candidatus* Phytoplasma aurantifolia' (16SrII group) (Caicedo et al. 2015). Our study allows to verify the presence of another phytoplasma enclosed in 16SrI-F subgroup. Phytoplasmas enclosed in this subgroup 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 in potatoes.

Past studies have shown that similar symptomatology has resulted in the detection of both '*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 ~~m~~ay transmit these pathogens in the field is necessary, along with a broader sampling in time and space.

Acknowledgments

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References

AGROCALIDAD (2015) Agencia Ecuatoriana de Aseguramiento de la Calidad del Agro. Informe técnico sobre punta morada en papa. Coordinación General de Sanidad Vegetal. Elaborado el 30 de octubre del 2015

Bertaccini A (2014) Phytoplasma Collection. International Phytoplasmologists Working Group web. http://www.ipwgnet.org/collection. Accessed 30 October 2017

Caicedo J, Crizón M, Pozo A, Cevallos A, Simbaña L, Rivera L, Arahana V (2015) First report of *'Candidatus* Phytoplasma aurantifolia' (16SrII) associated with potato purple top in San Gabriel-Carchi, Ecuador. New Dis Reprt 32

Castillo Carrillo C, Buitrón J, Insuasti M, Castillo N, Rivadeneira J, Cuesta X (2017) Avances en el diagnostic del agente causal del problema de punta morada en papa en Ecuador. Memorias del VII Congreso Ecuatoriano de la Papa. Tulcán, Ecuador

Crosslin JM, Lin H, Munyaneza JE (2011) Detection of '*Candidatus* Liberibacter solanacearum' in the potato psyllid, *Bactericera cockerelli* (Sulc), by conventional and real-time PCR. Southwest Entomol 36:125–135

Deng SJ, Hiruki C (1991) Amplification of 16S ribosomal-RNA genes from culturable and nonculturable mollicutes. J Microbiol Meth 14:53–61

Ferreira ME, Grattapaglia D (1998) Introducción al uso de marcadores moleculares en el análisis genético. EMBRAPA (Empresa Brasileira de Pesquisa Agropecuária) - CENARGEN (Centro Nacional de Pesquisa de Recursos Genéticos e Biotecnologia

Gutiérrez-Ibáñez AT, Sánchez-Pale JR, Cerda AL, Ramírez DJF, Balbuena AM, Alvarado MA (2013) Detección de '*Ca.* Liberibacter solanacearum' y fitoplasmas en cultivo de papa (*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

229 Santos-Cervantes ME, Chávez-Medina JA, Acosta-Pardini J, Flores-Zamora GL, Mendez-
230 Lozano J, Leyva-Lopez NE (2010) Genetic diversity and geographical distribution of
231 phytoplasmas associated with potato purple top disease in Mexico. Plant Dis 94:388-395
232 Satta E, Ramirez AS, Paltrinieri S, Contaldo N, Benito P, Poveda JB, Bertaccini A 2017
233 Simultaneous detection of mixed '*Candidatus* Phytoplasma asteris' and '*Ca*. Liberibacter
234 solanacearum'infection in carrot. Phytopath Medit 55~~(3)~~:401-409
235 Schneider B, Seemüller E, Smart CD, Kirkpatrick BC (1995) Phylogenetic classification of
236 plant pathogenic mycoplasma-like organisms or phytoplasmas. In: Razin S, and
237 Tully JG (eds) Molecular and diagnostic procedures in mycoplasmology
238 Academic Press, San Diego
239 Tamura K, Nei M (1993) Estimation of the number of nucleotide substitutions in the
240 control region of mitochondrial DNA in humans and chimpanzees. Mol Biol and Evol
241 10:512-526
242 Tamura K, Stecher G, Peterson D, Filipski A, Kumar S (2013) MEGA6: Molecular
243 Evolutionary Genetics Analysis version 6.0. Mol Biol and Evol 30:2725-2729

Table 1. Nested PCR detection of phytoplasma in potato from 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

[a] Sequenced samples

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.

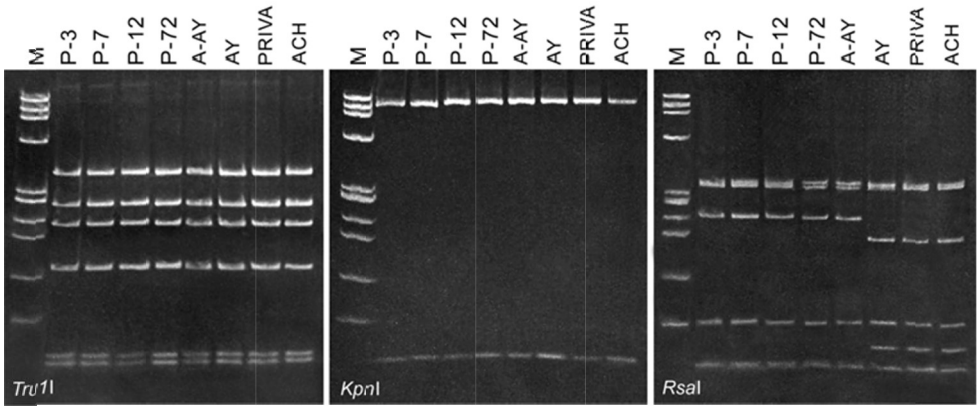
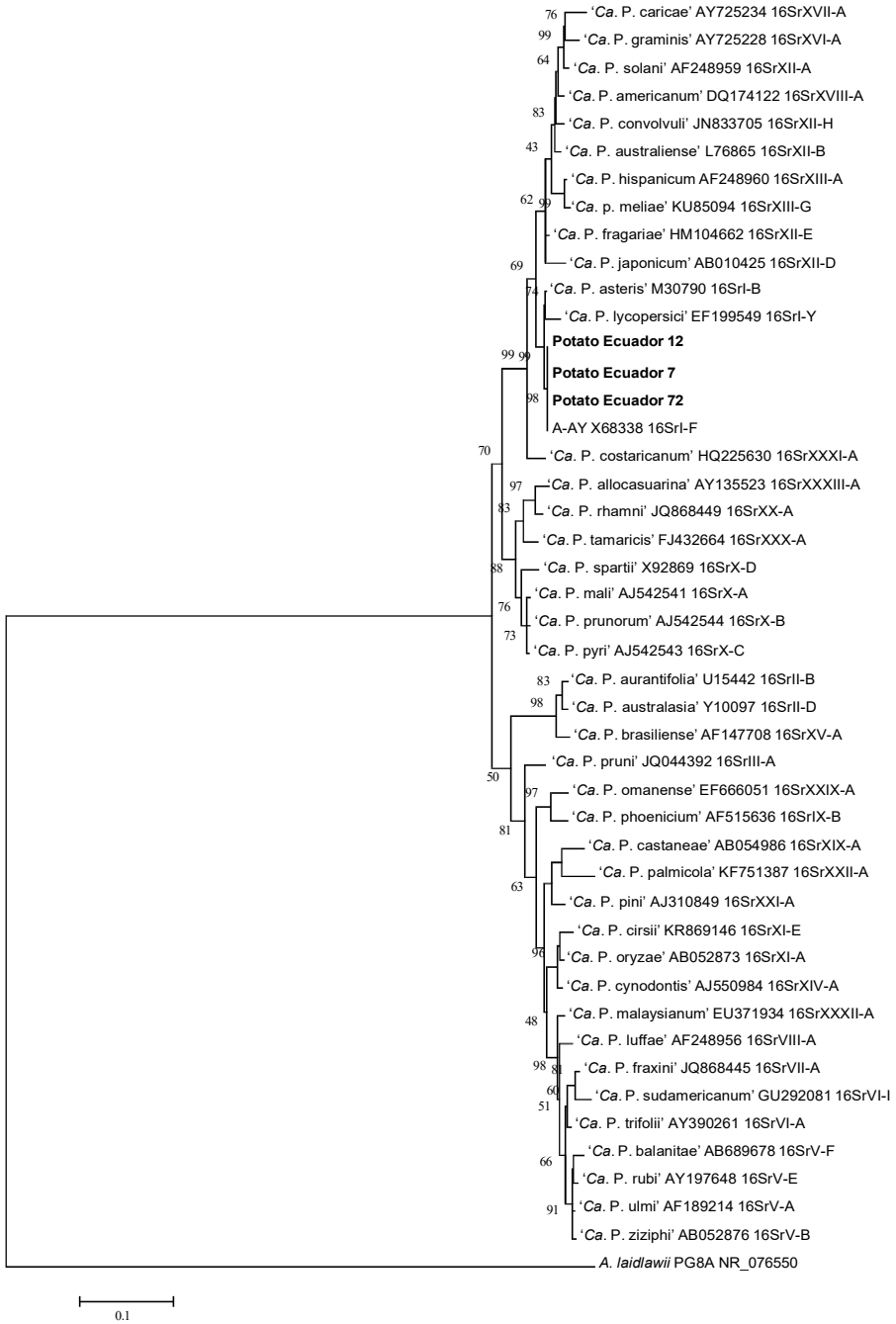


Figure. 2. Polyacrylamide gels 6.7% showing the restriction fragment length polymorphism patterns of R16(I)F1/R1 amplicons from phytoplasma strains from potatoes (P-3, P-7. P12, P72) and controls (A-AY, 16SrI-F; AY, 16SrI-B; PRIVA, 16SrI-L, and ACH, 16SrI-C), the restriction enzymes employed are indicated 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

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.