1 Title: '***Candidatus* Phytoplasma platensis**', a novel taxon associated with daisy
2 (***Bellis perennis***) virescence and related diseases in South America

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11 **running title:** "*Candidatus* Phytoplasma platensis"

12 GenBank [EMBL/DDBJ] accession numbers of gene sequence from representative
13 strain ('Ca. Phytoplasma platensis' BellVir) are: MK135798, KC412019; MK140657,
14 MG435348, MG435349 (16S rRNA, rplV-rps3, secA, imp and idpA respectively).

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23 Abstract

24 Bellis perennis virescence (BellVir) phytoplasma affects ornamental daisies in
 25 Argentina. It has been previously classified within the X-disease group, subgroup III-J,
 26 which is one of the most important and widely distributed in South America, affecting
 27 diverse plant hosts. In this study, we compared 16S rRNA, ribosomal proteins rplV and
 28 rps3, secA and immunodominant proteins imp and idpA genes of BellVir phytoplasma
 29 with previously described '*Candidatus* Phytoplasma' species. The 16S rRNA gene of
 30 strain BellVir shared less than 97.5% with all previously described '*Ca. Phytoplasma*'
 31 taxa except for '*Ca. Phytoplasma pruni*'. According to the recommended rules for the
 32 description of novel taxa within '*Ca. Phytoplasma*', it should be considered as '*Ca. P.*
 33 *pruni*'-related strain. However, multilocus analysis showed further molecular diversity
 34 that distinguished BellVir phytoplasma from '*Ca. Phytoplasma pruni*'. Besides, BellVir
 35 phytoplasma and 16SrIII-J related strains have a geographical distribution restricted to
 36 South America, where '*Ca. P.pruni*' has not been detected. Two insect vectors have
 37 been reported to transmit 16SrIII-J phytoplasmas, which have not been found to
 38 transmit '*Ca. Phytoplasma pruni*'. Having a wide host range, they have not been
 39 detected in *Prunus persica*. Therefore, based on multilocus sequence analyses,
 40 specific vector transmission and geographical distribution, we propose the recognition
 41 of the novel phytoplasma species '*Ca. Phytoplasma platensis*', within the X-disease
 42 clade, with Bellis perennis virescence phytoplasma as the reference strain.

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45 The provisional genus '*Candidatus* Phytoplasma', formally described in 2004,
 46 includes plant pathogenic non-helical *Mollicutes* that inhabit plant phloem and insects
 47 [1]. Phytoplasmas have been reported worldwide causing diseases in more than 1000
 48 plant species [2]. In plant hosts they colonize mainly sieve tube elements but can reach

parenchymatic companion cells. They are transmitted among plant hosts by sucking insects, such as leafhoppers and psyllids, in a persistent manner and could be detected in the insects' gut, hemolymph, salivary glands and other organs [3]. Symptomatic plants can show stunting, die back, abnormal shoot proliferation (witches' broom), leaf yellowing or reddening, reduced leaf size and deformation. Other symptoms include flower sterility, phyllody and virescence [4, 5].

The provisional status of *Candidatus* responds to the difficulty of obtaining pure cultures, and consequently phenotypic description. Instead, conserved gene sequence analysis has been used for the detection and classification of phytoplasma strains. Based on 16S rRNA gene sequence and RFLP analysis phytoplasmas have been classified into 16Sr groups and subgroups [6, 7, 8]. So far, more than thirty groups have been described, most of which host at least one 'Ca. Phytoplasma species' [9, 10]. Further distinction can be achieved by including biological features such as plant hosts, symptoms, insect vectors, geographical distribution, and multilocus sequence analysis [11–14]. According to the recommended rules for the description of novel taxa within 'Ca. Phytoplasma', a new species should refer to a unique 16S rRNA gene sequence with $\leq 97.5\%$ similarity with a previously described 'Ca. Phytoplasma species'. However, a strain that has higher sequence similarity could be described as a new species if it has clearly different biological characteristics including host range and vector transmission, and significative molecular diversity [1].

Bellis perennis virescence phytoplasma (BellVir) (Figure S1) was first reported in 2013 affecting ornamental daisies in Argentina. Based on 16S rRNA and ribosomal protein genes analyses it has been classified within group 16SrIII or X-disease group, subgroup 16SrIII-J [15]. Chayote witches' broom (ChWBIII) phytoplasma was the first 16SrIII-J strain detected in Brazil [16]. After that, subgroup 16SrIII-J phytoplasmas have been reported in various cultivated, domesticated and wild plant hosts in South America, mainly Argentina, Southern Brazil and Chile [15, 17–21]. Infected plant hosts

76 showed diverse symptoms including leaf size and general growth reduction, internode
77 shortening and shoot proliferation, color changing, phyllody and virescence. The X-
78 disease or 16SrIII is one of the most numerous and diverse groups, composed by more
79 than 25 subgroups. Up to now, one ‘*Ca. Phytoplasma*’ species has been described
80 (‘*Ca. Phytoplasma pruni*’) that includes strains of group 16SrIII, with Peach X-disease
81 phytoplasma from subgroup 16SrIII-A as reference strain (PX11CT1, JQ044392).
82 Phytoplasmas from other 16SrIII subgroups have been considered as related strains
83 since they show differences in the oligonucleotide unique regions of the rRNA gene
84 [22]. However, the high diversity within 16SrIII group led the authors to suggest that the
85 analyzed 16SrIII group phytoplasma lineages might represent at least two, or even
86 three different phytoplasma taxa..

87 *Bellis perennis* phytoplasma (BellVir, 16SrIII-J) [15] was selected as reference
88 strain for molecular analyses. The phytoplasma was first transmitted from infected
89 daisies to healthy periwinkle (*Catharanthus roseus* (L.) G. Don.) using *Cuscuta*
90 *subinclusa*. Once the infection was established in periwinkle, the strain was
91 perpetuated by periodical grafting. Typical symptoms of phyllody, virescence, leaf size
92 reduction and yellowing were observed in infected plants 3 to 6 months after grafting
93 (Figure S1). For PCR amplifications, genomic DNA was extracted from BellVir infected
94 periwinkle leaves and petioles by the CTAB technique [23]. Phytoplasma 16S rRNA
95 gene was amplified by PCR using primers P1/P7 [24] according to previously described
96 protocol [15]. Partial secA gene amplification (~0.80kb) was performed using
97 SecAfor1/SecArev3 primer pair following the conditions proposed by [25]. For
98 immunodominant proteins, we designed specific primers based on the genomic
99 information available in public database. So far, only one draft genome has been
100 described for subgroup 16SrIII-J [26] which belongs to Vc33 phytoplasma isolate from
101 periwinkle. Annotation pipeline led us to identify imp and idpA gene sequences [27].
102 Two new sets of primers impXd-Fw1/impXd-Rv1 and idpAXd-Fw1/idpA-Rv1 were

designed in order to amplify by PCR a genomic fragment containing the complete sequence of *imp* and *idpA* genes, respectively (Table S3, Supplementary material). The amplicons were purified and cloned as described previously [28]. Three clones for each isolate were bidirectionally sequenced using an automated DNA Sanger sequencer (Unidad Genómica, Instituto de Biotecnología-Instituto Nacional de Tecnología Agropecuaria, Argentina). Final consensus sequences (3X coverage) were assembled using the Geneious R10 software and deposited in the GenBank nucleotide database. The phylogenetic reconstruction for each gene was performed using Maximum Likelihood method from the MEGA 6 software package [29].

BellVir represents a novel taxon for the provisional genus ‘Candidatus Phytoplasma’

The signature sequence (5'-CAAGAYBATKATGKTAGCYGGDCT-3') characteristic of the provisional genus ‘*Candidatus* Phytoplasma’ is contained in BellVir’s 16S rRNA gene (5'-CAAGACTATGATGTGTAGCTGGACT-3') (263-287). The 16S rRNA gene (MK135798) of strain BellVir shared less than 97.5% with corresponding fragments of the 16S rRNA genes from all previously described ‘*Ca. Phytoplasma*’ taxa except for ‘*Ca. Phytoplasma pruni*’, with 98.79-98.87% nucleotide sequence identity (Table 1). However, multilocus analysis showed further molecular diversity that distinguished BellVir phytoplasma from ‘*Ca. Phytoplasma pruni*’. Besides, BellVir phytoplasma and 16SrIII-J related strains have a geographical distribution restricted to southern South America and have particular biological characteristics. Having a wide host range that includes *Bellis perennis* (used as reference strain), *Allium sativum*, *Cucurbita maxima*, *Coffea arabica*, *Solanum lycopersicum*, *S. melongea*, *Helianthus annuus*, *Sechium edule*, *Brassica oleracea*, *Beta vulgaris*, *Fragaria x annanasa*, *Lactuca sativa*, *Manihot sculenta* and *Prunus avium* (cherry) among others[16, 20, 29–31, 33], phytoplasmas from this subgroup have not been detected in *Prunus persica* (peach). As regards vector transmission, two insect vectors

130 have been reported to transmit 16SrIII-J phytoplasmas, *Paratanus exitiosus* and
131 *Bergallia valdiviana*, which have not been found to transmit ‘Ca. Phytoplasma pruni’
132 [19, 34]. ‘Ca. Phytoplasma pruni’ has not been detected until now in South American
133 countries and, if 16SrIII-J phytoplasmas were considered related to it, attempts to
134 regulate the pathogen introduction into these countries would be very difficult to
135 accomplish.

136 The phylogenetic tree based on 16S rDNA sequences of BellVir phytoplasma
137 and known ‘Ca. phytoplasma species’ showed that BellVir clustered with ‘Ca.
138 Phytoplasma pruni’ but separated into an independent branch within the cluster (Figure
139 1). A broader phylogenetic analysis showed that ‘Ca. Phytoplasma platensis’ and ‘Ca.
140 Phytoplasma pruni’ strains conform two well defined clades (Figure S2, supplementary
141 material). Previous works had shown the same topology, separating 16SrIII-J
142 phytoplasmas from other 16SrIII subgroups [15, 18, 21]. When the unique signature
143 regions that distinguish ‘Ca. Phytoplasma pruni’ were examined in BellVir’s 16S rRNA
144 gene sequence, 7 out of the 13 unique regions showed at least one nucleotide
145 difference between them (Figure S3, supplementary material).

146 **Multilocus sequence analyses differentiate ‘*Candidatus Phytyoplasma platensis*’** 147 **from ‘*Candidatus Phytoplasma pruni*’**

148 Other genomic regions were examined to determine molecular diversity of
149 BellVir comparing with previously described ‘Ca. Phytoplasma pruni’. Sequences of
150 ribosomal protein genes rplV and rps3 of BellVir showed >98.5% identity with related
151 strains and 96.5% with ‘Ca. Phytoplasma pruni’. RFLP patterns generated by restriction
152 enzymes *AluI* and *DraI* distinguished BellVir from ‘Ca. Phytoplasma pruni’ [15]. The
153 phylogenetic tree showed that BellVir and related phytoplasmas integrate a cluster
154 separated from ‘Ca. Phytoplasma pruni’ and related strains (Figure 2). Multiple
155 alignment of ribosomal proteins gene sequences revealed the presence of 17 SNPs

156 that distinguish ‘*Ca. Phytoplasma platensis*’ strains from the ‘*Ca. Phytoplasma pruni*’
157 strains (Table S2, supplementary material). Similar results were obtained when *secA*
158 gene was analyzed, and the amino acid sequence corresponding to BellVir *secA*
159 protein (MK140657) showed 99.4% identity with Vac33 strain (LLKK01000003) while
160 both of them shared a maximum identity of 96.6% with ‘*Ca. Phytoplasma pruni*’ strains.
161 The resulting tree had the same topology as the generated by 16S rRNA and ribosomal
162 protein genes, supporting the separation of BellVir phytoplasma (Figure 3).
163 Immunodominant proteins would not be correlated with that of 16Sr DNA; however, *imp*
164 is a gene well conserved over a wide range of phytoplasmas and can reflect in
165 between phytoplasma differences regarding host range and vector transmission [35].
166 Phytoplasmas immunodominant proteins have been classified into three distinct types:
167 (i) immunodominant membrane protein (*Imp*); (ii) immunodominant membrane protein
168 A (*IdpA*); and (iii) antigenic membrane protein (*Amp*) [36]. BellVir phytoplasma has the
169 same type of immunodominant membrane proteins as all 16SrIII-group phytoplasmas
170 since both *imp* and *idpA* genes could be amplified [37]. BellVir *imp* amino acid
171 sequence had 97.7% identity with 16SrIII-J phytoplasma Vac33, and 58.3-61% identity
172 with ‘*Ca. Phytoplasma pruni*’ related strains, which showed 97.7-79.6% identity among
173 them. The phylogenetic tree constructed with *imp* aa sequences of 16SrIII
174 phytoplasmas clearly separated BellVir from ‘*Ca. Phytoplasma pruni*’ and related
175 strains (Figure S4, supplementary material). Similar situation arise within the *idpA*
176 protein, since the highest identity occurs with Vac33 phytoplasma (87.4%) and 66.26-
177 69.51% identity *Ca. Phytoplasma pruni*’ related strains, which showed 99.65-63.61%
178 identity among them. The topology of the phylogenetic tree constructed with *idpA* aa
179 sequences resembled those of *imp*, and showed once again a clear separation of ‘*Ca.*
180 *Phytoplasma platensis*’ and ‘*Ca. Phytoplasma pruni*’ clades.

181 Based on multilocus sequence analyses, specific vector transmission and
182 geographical distribution, we propose the recognition of the new phytoplasma species

183 'Ca. Phytoplasma platensis', within the X-disease clade. The continuous advance in the
184 field of genomics and the fundamental biology of phytoplasmas will allow us to describe
185 new phytoplasma species.

186 **Description of '*Candidatus* Phytoplasma platensis'**

187 '*Candidatus* Phytoplasma platensis' (pla. ten'. sis. L. masc. adj. referring to Río de la
188 Plata, a river representative of Argentina and southern South America, where the
189 reference strain was identified).

190 [(Mollicutes) NC; NA; O, wall less; NAS (GenBank accession number XXXX),
191 oligonucleotide sequences of unique regions of the 16S rRNA gene; 5'-617-
192 CTATAGAACTGTTTTACTAGAGTGAGTTAGAGGCAAG-654-3' (*Bellis perennis*,
193 phloem); MJ].

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197 **Conflicts of interest**

198 The authors declare that there are no conflicts of interest

199 **Ethical statement**

200 No humans or animals were subjects in this work.

201

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327 **Figure Legends**

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329 **Figure 1:** Phylogenetic tree inferred from 16S rRNA gene sequence analysis using the
 330 Maximum Likelihood method implemented in the Molecular Evolutionary Genetics
 331 Analysis program (MEGA 6). *Acholeplasma palmae* was used as outgroup. The
 332 numbers on the branches are bootstrap (confidence) values (expressed as percentage
 333 of 1000 replicates). GenBank accession number for each taxon is given between
 334 parentheses. The final tree includes reference strain of 48 previously described or
 335 incidentally cited as 'Ca. Phytoplasma species' and 'Ca. Phytoplasma platensis'
 336 (marked with black diamond). Bar, number of nucleotide substitutions per site.

337 **Figure 2:** Phylogenetic tree inferred from rplV and rps3 genes sequence analysis using
 338 the Maximum Likelihood method implemented in the Molecular Evolutionary Genetics
 339 Analysis program (MEGA 6). The numbers on the branches are bootstrap (confidence)
 340 values (expressed as percentage of 1000 replicates). GenBank accession number for
 341 each taxon is given between parentheses. R: reference strains. Bar, number of
 342 nucleotide substitutions per site.

343 **Figure 3:** Phylogenetic tree inferred from secA aa sequence analysis using the
 344 Maximum Likelihood method implemented in the Molecular Evolutionary Genetics
 345 Analysis program (MEGA 6). The numbers on the branches are bootstrap (confidence)
 346 values (expressed as percentage of 1000 replicates). GenBank accession number for
 347 each taxon is given between parentheses. Sequence of 'Ca. platensis' obtained in this
 348 work is marked with black diamond. Bar, number of nucleotide substitutions per site.

349