

## '*Candidatus Phytoplasma convolvuli*', a new phytoplasma taxon associated with bindweed yellows in four European countries

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Plants of *Convolvulus arvensis* exhibiting symptoms of undersized leaves, shoot proliferation and yellowing, collectively defined as bindweed yellows, were sampled in different regions of Europe and assessed for phytoplasma infection by PCR amplification using phytoplasma universal rRNA operon primer pairs. Positive results were obtained for all diseased plants. RFLP analysis of amplicons comprising the 16S rRNA gene alone or the 16S rRNA gene and 16–23S intergenic spacer region indicated that the detected phytoplasmas were distinguishable from all other previously described rRNA gene sequences. Analysis of 16S rRNA gene sequences derived from seven selected phytoplasma strains (BY-S57/11, BY-S62/11, BY-I1015, BY-I1016, BY-BH1, BY-BH2 and BY-G) showed that they were nearly identical (99.9–100% gene sequence similarity) but shared less than 97.5% similarity with comparable sequences of other phytoplasmas. Thus, BY phytoplasmas represent a new taxon whose closest relatives are stolbur phytoplasma strains and '*Candidatus Phytoplasma fragariae*' with which they share 97.2% and 97.1% 16S rRNA gene sequence similarity, respectively. Phylogenetic analysis of 16S rRNA gene sequences confirmed that bindweed yellows phytoplasma strains collectively represent a distinct lineage within the phytoplasma clade and share a common ancestor with previously published or proposed '*Candidatus Phytoplasma*' taxa within a major branch including aster yellows and stolbur phytoplasmas. On the basis of unique 16S rRNA gene sequences and biological properties that include a single host plant species and a geographical distribution limited to parts of Europe, the bindweed yellows (BY) phytoplasmas represent a coherent but discrete taxon, '*Candidatus Phytoplasma convolvuli*', with strain BY-S57/11 (GenBank accession no. JN833705) as the reference strain.

**Abbreviations:** BY, bindweed yellows; SNP, single nucleotide polymorphism.

The GenBank/EMBL/DDBJ accession number for the 16S rRNA gene sequence and the 16S–23S rRNA gene spacer region of '*Candidatus Phytoplasma convolvuli*' strain BY-S57/11 is JN833705. The GenBank/EMBL/DDBJ accession numbers for the 16S rRNA gene sequences of '*Candidatus Phytoplasma convolvuli*'-related strains BY-S62/11, BY-G, BY-BH1, BY-BH2, BY-I1015 and BY-I1016 are JN833706, JN833707, JN833708, JN833709, JN833710 and JN833711, respectively.

Phytoplasmas are plant-pathogenic bacteria that are associated with diseases in more than 1000 plant species (Bertaccini, 2007) exhibiting an array of symptoms that suggests profound disturbances in the normal balance of growth regulators. Phytoplasmas belong to the class *Mollicutes*, included in the recently introduced phylum *Tenericutes* (Brown, 2010) on the grounds of their lack of rigid cell walls and analyses of strongly supported universal phylogenetic markers as an alternative to 16S rRNA gene sequence analysis. These prokaryotes are transmitted by

insects belonging to the families *Cicadellidae*, *Cixidae*, *Psyllidae*, *Delphacidae* and *Derbidae* (Weintraub & Beanland, 2006).

Molecular analyses have provided considerable insight into the diversity and genetic interrelationships of phytoplasmas (Hogenhout *et al.*, 2008). RFLP analysis of 16S rRNA gene sequences has been widely used as a means to identify, differentiate and classify phytoplasmas into a series of groups and subgroups (Lee *et al.*, 1998; reviewed by Bertaccini & Duduk, 2009). Phylogenetic analysis of 16S rRNA gene sequences has also provided a basis for a formal taxonomy of the phytoplasmas according to guidelines proposed by the International Program for Comparative Mycoplasma/Spiroplasma Working Team's Phytoplasma Taxonomy Group (IRPCM, 2004). At the time of writing, the provisional status of 'Candidatus Phytoplasma' has been assigned to 30 phytoplasmas (Davis *et al.*, 2011; Lee *et al.*, 2011; Malembic-Maher *et al.*, 2011; reviewed by Bertaccini & Duduk, 2009).

*Convolvulus arvensis* (bindweed), is a wild plant species belonging to the family Convolvulaceae, and it is a widespread host of stolbur phytoplasmas (16SrXII-A) mainly in the European vineyard ecosystems (Maixner, 1994; Sforza *et al.*, 1998). Symptomatic plants show undersized leaves, shoot proliferation and sometimes yellowing. However, in a number of cases these symptoms were associated with phytoplasmas that could not be assigned to previously described taxa nor established 16S rRNA groups/subgroups (Marcone *et al.*, 1997; Seemüller *et al.*, 1998; Martini *et al.*, 2008).

From 2004 to 2011, a number of symptomatic bindweed samples (BY) and four symptomless plants were collected from different locations in four European countries (Italy, Serbia, Bosnia & Herzegovina and Germany). After preliminary screening for phytoplasma presence, a total of nine symptomatic samples (Italy: BY-I1006, BY-I1015, BY-I1016; Serbia: BY-S57/11, BY-S62/11, BY-S63/11; Bosnia & Herzegovina: BY-BH1, BY-BH2 and Germany: BY-G) were selected for phytoplasma molecular characterization.

Total nucleic acid extraction from symptomatic and asymptomatic samples was performed using a CTAB plant DNA extraction procedure (Doyle & Doyle, 1990). PCR was carried out using universal primer pairs P1/16S-SR or P1/P7 (Deng & Hiruki, 1991; Lee *et al.*, 2004; Schneider *et al.*, 1995; Smart *et al.*, 1996), which amplified an approximately 1550 bp product corresponding to the phytoplasma whole 16S rRNA gene and partial 16S–23S rRNA spacer region, or an approximately 1800 bp product from the 5' end of the 16S rRNA gene to the 5' end of the 23S rRNA gene, including the spacer region, respectively. Direct PCR assays were also performed with primer pair R16F2n/R2 (Gundersen & Lee, 1996), which amplified the 16S rRNA gene alone (approximately 1200 bp). P1/P7 and R16F2n/R2 PCR products were analysed by single-enzyme digestion with *AluI*, *HaeIII*, *HhaI*, *HinfI*, *HpaII*, *MboI*, *TaqI* and *TruI*. The resulting RFLP patterns were compared

with those of the stolbur phytoplasma P-TV reference strain maintained in periwinkle at the University of Udine, Italy, and with previously published phytoplasma strains (Lee *et al.*, 1998).

To obtain near full-length 16S rRNA gene sequences of selected representative strains, P1/16S-SR amplicons from five samples (BY-I1015, BY-I1016, BY-BH1, BY-BH2 and BY-G) were purified using the Wizard SV Gel and the PCR Clean-Up System kit (Promega), cloned in *Escherichia coli* using the pGEM-T Easy Vector (Promega) according to the manufacturer's instructions and sequenced by a commercial service (ENEA, Italy). P1/P7 amplicons from two other samples (BY-S57/11 and BY-S62/11) were purified using mi-PCR Purification kit (Metabion) and directly sequenced by a commercial service (Macrogen, Korea). Sequences were assembled using the Staden software package (Staden *et al.*, 2000), aligned using CLUSTAL\_X (Thompson *et al.*, 1997), and searched for single nucleotide polymorphisms (SNPs) using the BioEdit program (Hall, 1999). The nucleotide sequences were deposited in GenBank with accession numbers JN833705 (strain BY-S57/11), JN833706 (strain BY-S62/11), JN833707 (strain BY-I1015), JN833708 (strain BY-I1016), JN833709 (strain BY-BH1), JN833710 (strain BY-BH2) and JN833711 (strain BY-G).

Strain BY-S57/11, designated as the reference strain for all molecular analyses, was successfully maintained in naturally infected *C. arvensis* shoots by micropropagation as part of the phytoplasma collection located at the University of Bologna (Bertaccini, 2010).

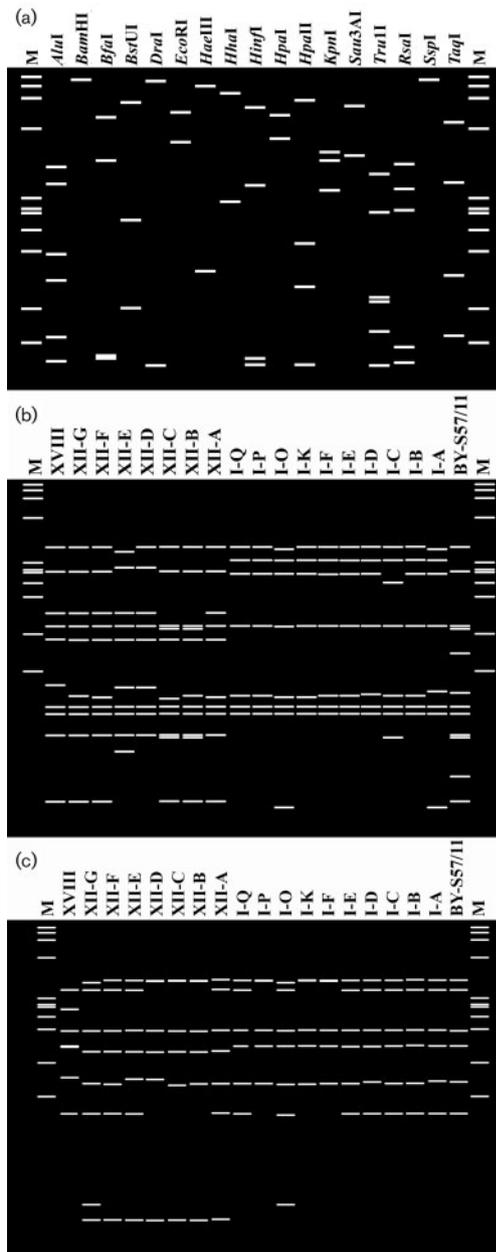
Collective RFLP patterns of strain BY-S57/11 and coefficients of similarity using 17 restriction enzymes were generated by virtual gel analysis using *iPhyClassifier* software (Zhao *et al.*, 2009).

The 16S rRNA gene sequences of strain BY-S57/11 and most closely related phytoplasmas were compared by virtual RFLP analyses using pDRAW32 software ([www.acaclone.com](http://www.acaclone.com)).

The 16S rRNA gene sequences of strain BY-S57/11 along with 31 published or suggested 'Candidatus Phytoplasma' taxa (IRPCM, 2004; reviewed by Duduk & Bertaccini, 2011; Davis *et al.*, 2011) were aligned using the CLUSTAL W program in the MEGA5 software package (Tamura *et al.*, 2011). The maximum-parsimony tree was obtained using the Close-Neighbour-Interchange algorithm in the MEGA5 software package, with search level 3 in which the initial trees were obtained with the random addition of sequences (10 replicates). *Acholeplasma laidlawii* (GenBank accession no. U14905) was used as an outgroup taxon to root the tree. The analysis was replicated 2000 times. Bootstrapping was performed to estimate the stability and support for the inferred clades.

All nine plants tested positive for phytoplasma infection by PCR. No amplification was observed when total nucleic acids from the four asymptomatic plants were used as templates. RFLP analyses of the PCR-amplified 16S rRNA





**Fig. 2.** Collective virtual RFLP patterns (a) derived from *in silico* RFLP analysis, using iPhyClassifier, of 16S rRNA gene (R16F2n/R2 amplicon) fragments from bindweed yellows phytoplasma strain BY-S57/11 with 17 restriction enzymes: *AluI*, *BamHI*, *BfaI*, *BstUI*, *DraI*, *EcoRI*, *HaeIII*, *HhaI*, *HinfI*, *HpaI*, *HpaII*, *KpnI*, *Sma3AI*, *Tru1I*, *RsaI*, *SspI*, and *TaqI*. M, marker øX174 RFI DNA *HaeIII* digest. Virtual RFLP patterns derived from *in silico* RFLP, using pDRAW32, with the two key restriction enzymes: *Tru1I* (b) and *AluI* (c) distinguishing bindweed yellows phytoplasma strain BY-S57/11 from representative strains of groups 16SrI and 16SrXII. The 16S subgroups are represented by the following strains (GenBank accession numbers and strain designations are in parentheses): I-A (NC\_007716, strain AY-WB), I-B (NC\_005303, OY-M and M30790, 'Ca. P. asteris'), I-C (AF222065, CPh). I-D (AY265206, PaWB), I-E (AY265213, BBS3), I-F (AY265211, ACLR-AY), I-K (U96616, STRAWB2), I-O (AF268405, aster yellows phytoplasma O), I-P (HM590626, PopD), I-Q (AY034089, ChLL), XII-A (AJ964960, STOL), XII-B (L76865, 'Ca. P. australiense'), XII-C (AJ243045, strawberry lethal yellows), XII-D (AB010425, 'Ca. P. japonicum'), XII-E (DQ086423, 'Ca. P. fragariae'), XII-F (EU836652, BN-Op30), XII-G (EU836647, strain BN-Fc3), and XVIII-A (DQ174122, 'Ca. P. americanum'). The restriction fragments were resolved by *in silico* electrophoresis through 3% agarose gel. M, marker øX174 RFI DNA *HaeIII* digest.

aster yellows and stolbur phytoplasmas on the phylogenetic tree (Fig. 3).

Sequence similarity comparisons revealed that the 16S rRNA gene sequence of strain BY-S57/11 shares less than 97.5% sequence identity with any of the previously described 'Ca. Phytoplasma' taxa. Therefore, according to guidelines for assigning incompletely described prokaryotes to the provisional status of 'Candidatus' (Murray & Stackebrandt, 1995) and to recommendations by the IRPCM (2004), the phytoplasma associated with undersized leaves, shoot proliferation, and yellowing of *C. arvensis* in Europe represents a new 'Ca. Phytoplasma' taxon. On the basis of unique 16S rRNA gene sequences and biological properties represented by the single plant host, the BY phytoplasma is proposed to be designated 'Candidatus Phytoplasma convolvuli' with the following description.

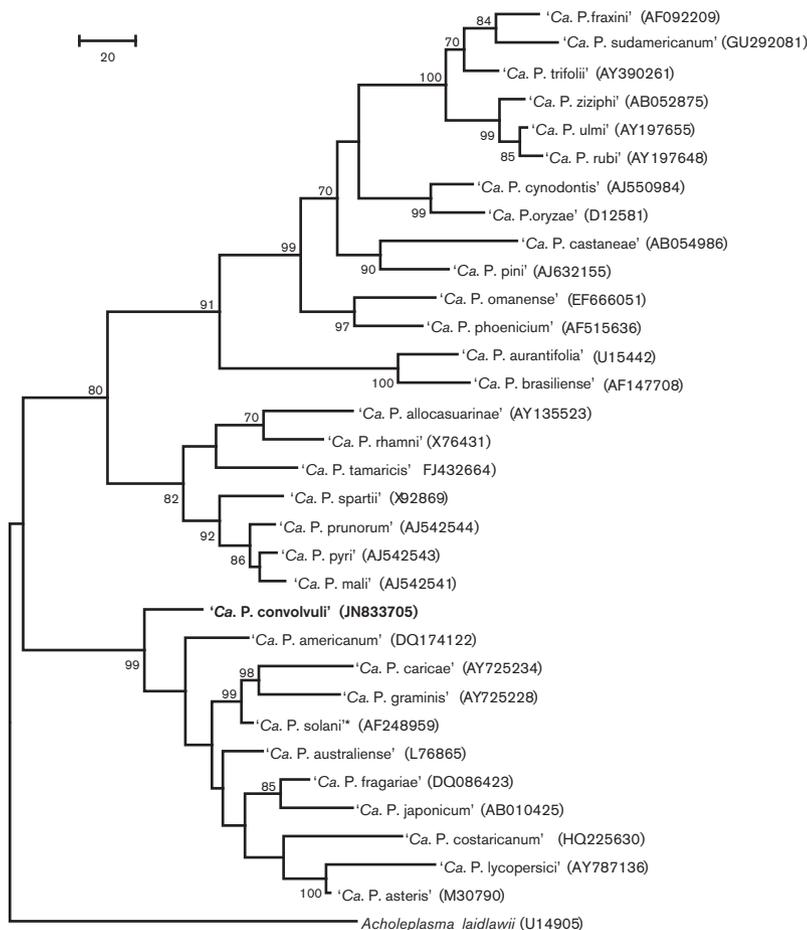
**Description of 'Candidatus Phytoplasma convolvuli'**

'Candidatus Phytoplasma convolvuli' (con.vol'vu.li. L. gen. n. *convolvuli* of bindweed, isolated from *Convolvulus arvensis*; epithet referring to the plant host).

Reference strain is BY-S57/11 (GenBank accession no. JN833705). Related phytoplasma strains include BY-S62/11, BY-G, BY-BH1, BY-BH2, BY-I1015 and BY-I1016, associated with bindweed yellows (BY) disease; (GenBank accession numbers JN833706, JN833707, JN833708, JN833709, JN833710 and JN833711). The vector(s) that transmits this phytoplasma is unknown. [(Mollicutes) NC; NA; O, wall-less; NAS (GenBank accession no. JN833705),

<sup>139</sup>5'-GATAGGAAGGCATCTTCTTG-3'<sup>158</sup>, <sup>788</sup>5'-ACGT-TGGTTAAACCA-3'<sup>803</sup>, and <sup>949</sup>5'-GCTTTTGCAAAGC-TT-3'<sup>963</sup>, which differ from the corresponding regions in the 16S rRNA genes of all previously described or proposed 'Ca. Phytoplasma' taxa.

Phylogenetic analysis using nearly full-length 16S rRNA gene sequence of strain BY-S57/11, and of 31 published or suggested 'Ca. Phytoplasma' taxa yielded five equally parsimonious trees; one of these trees is presented in Fig. 3. Numbers on the branches are bootstrap values obtained for 2000 replicates. The phylogenetic analysis indicated that strain BY-S57/11 represents a distinct lineage and shares a common ancestor with previously published or suggested 'Ca. Phytoplasma' taxa within a major branch including



**Fig. 3.** Phylogenetic tree constructed by parsimony analysis of near full-length 16S rRNA gene sequences from previously described or suggested 'Ca. Phytoplasma' taxa and the novel 'Ca. P. convolvuli' phytoplasma strain BY-S57/11. \*, 'Candidatus' name proposed at the Xth International Congress of the International Organization of Mycoplasmatology held in 1994 in Bordeaux, France, but not yet formally described, reported here as incidental citations which do not constitute prior citations, according to rule 28b of the Bacteriological Code (Lapage *et al.*, 1992). *Acholeplasma laidlawii* was used as an outgroup taxon to root the tree. Branch lengths are proportional to the number of inferred character state transformations. Bootstrap values (measures of support for the inferred subclades) are shown on branches. GenBank accession numbers are given in parentheses. Bar, 20 inferred character state changes.

oligonucleotide sequences of unique regions of the 16S rRNA gene are 5'-GCCTTTGGGC-3' (41–50), 5'-GATAGGAAGGCATCTTCTTG-3' (139–158), 5'-ACGTTGGTTAAAACCA-3' (788–803), 5'-GCTTTTGCAAAGCTT-3' (949–963); P (*Convolvulus arvensis*, phloem); M]. Martini *et al.*, this study. As described previously (IRPCM, 2004), specific nucleotide signatures: A at position 242, T at position 286 and T at position 1247 (in the sequence with GenBank accession no. M30790), that are characteristic of 'Ca. Phytoplasma' are present in the sequence of 'Ca. P. convolvuli' strains BY-S57/11, BY-S62/11, BY-I1015, BY-I1016, BY-BH1, BY-BH2 and BY-G.

## Acknowledgements

Part of this work was supported by the Ministry of Education and Science, Republic of Serbia, grant number TR31043. We thank Martina Stark-Urnau for providing bindweed samples from Germany.

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