

# A novel subgroup 16SrVII-D phytoplasma identified in association with erigeron witches' broom

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*Erigeron* sp. plants showing symptoms of witches' broom and stunting were found near orchards of passion fruit in São Paulo state, Brazil. These symptoms were indicative of infection by phytoplasmas. Thus, the aim of this study was to detect and identify possible phytoplasmas associated with diseased plants. Total DNA was extracted from symptomatic and asymptomatic plants and used in nested PCR conducted with the primer pairs P1/Tint and R16F2n/16R2. Amplification of genomic fragments of 1.2 kb from the 16S rRNA gene confirmed the presence of phytoplasma in all symptomatic samples. The sequence identity scores between the 16S rRNA gene of the phytoplasma strain identified in the current study and those of previously reported '*Candidatus* Phytoplasma fraxini'-related strains ranged from 98 % to 99 % indicating the phytoplasma to be a strain affiliated with '*Candidatus* Phytoplasma fraxini'. The results from a phylogenetic analysis and virtual RFLP analysis of the 16S rRNA gene sequence with 17 restriction enzymes revealed that the phytoplasma strain belongs to the ash yellows phytoplasma group (16SrVII); the similarity coefficient of RFLP patterns further suggested that the phytoplasma represents a novel subgroup, designated 16SrVII-D. The representative of this new subgroup was named EboWB phytoplasma (*Erigeron bonariensis* Witches' Broom).

Phytoplasmas are cell-wall-less prokaryotes, causal agents of numerous diseases that occur in a diversity of botanical species, widely distributed throughout the world (Lee *et al.*, 2000). The taxonomy has been mainly based on the 16S rRNA gene and phytoplasmas are currently classified in distinct groups and subgroups, which are delineated on the basis of molecular characteristics and phylogeny (Lee *et al.*, 2010).

The first report, to our knowledge, of a representative of 16SrVII was made in the USA, in plants of the species *Fraxinus* and *Syringa* (Griffiths *et al.*, 1999). The phytoplasma was described as the reference strain of the putative species '*Candidatus* Phytoplasma fraxini' and was classified into subgroup A of the ash yellows phytoplasma group (16SrVII-A). In Chile, a phytoplasma of this subgroup was associated with grapevine yellows disease (Gajardo *et al.*, 2009). A phytoplasma presents in Canadian orchards, characterized from peach trees with symptoms of witches' broom, was identified as similar to the representative of

the 16SrVII-A subgroup (Zunnoon-Khan *et al.*, 2010). In Brazil, a 16SrVII-B phytoplasma was molecularly characterized in association with plants of *Erigeron* sp. and *Catharanthus roseus* that exhibited witches' broom (Barros *et al.*, 2002). In Argentina, a phytoplasma of the 16SrVII-B subgroup was also found in erigeron plants with witches' broom symptoms (Meneguzzi *et al.*, 2008). Also in Argentina, a phytoplasma present in alfalfa with symptoms of witches' broom was classified in a new subgroup 16SrVII-C (Conci *et al.*, 2005). Recently, in Brazil, a phytoplasma affiliated with this subgroup was identified in sunn hemp plants with shoot proliferation (Flôres *et al.*, 2013).

The species *Erigeron bonariensis* is native to South America and occurs abundantly in Argentina, Brazil, Colombia, Paraguay, Uruguay and Venezuela (Kissmann & Groth, 1999). This species is recognized as infesting abandoned areas, pasture fields, perennial crops (citrus and coffee) and annual crops (cotton, corn, soybeans and wheat) (Thébaud & Abbott, 1995). Numerous plants of *E. bonariensis* showing typical phytoplasma-induced symptoms were observed in areas adjacent to a passion fruit orchard, located in the municipality of Presidente Prudente, in the State of São Paulo, Brazil. In these areas the frequency of

The GenBank/EMBL/DDBJ accession number for the 16S rRNA sequence of the phytoplasma EboWB-Br01 is KJ831066.

A supplementary figure is available with the online Supplementary Material.

affected erigeron plants reached approximately 20 %. The affected plants were easily recognized by reduced size and the profuse production of short shoots, shortened internodes and abundant little leaves, generating a bushy and compact canopy (Fig. S1, available in the online Supplementary Material).

In the present study we demonstrate the constant association of a phytoplasma with the symptomatic erigeron plants and characterize molecularly this strain as representative of a novel 16SrVII-D subgroup.

Leaves and shoots were obtained from symptomatic (15 samples analysed) and asymptomatic (three samples analysed) plants grown in the same field. Total DNA was extracted from fresh tissues using a DNeasy Plant Mini kit (Qiagen). Maize bushy stunt phytoplasma, member of subgroup 16SrI-B (GenBank: AY265208) and DNA from asymptomatic erigeron plants represented positive and negative controls, respectively.

Detection of phytoplasma was performed using nested PCR assays with the universal phytoplasma primers P1/Tint (Deng & Hiruki, 1991; Smart *et al.*, 1996), followed by R16F2n/R16R2 (Gundersen & Lee, 1996). Amplified products were analysed after electrophoresis through 1 % agarose gels, Sybr Safe (Invitrogen) staining and visualization of DNA fragments in an UV transilluminator.

The fragments generated by nested PCR were cloned in *Escherichia coli* DH5 $\alpha$ , using the pGEM Easy Vector System I (Promega), and sequenced using the primer pair SP6/T7 (Malembic-Maher *et al.*, 2008). The sequences were aligned, compared with each other, with the sequences from phytoplasmas of different groups and with sequences of the members of distinct subgroups within the 16SrVII group. Nucleotide sequences were analysed using computer programs for reconstruction and sequence analysis (BioEdit, Phred phrap and Multiple Sequence Alignment – CLUSTAL W).

Virtual RFLP analysis was simulated in a computer based on all distinct representatives of the subgroups within of the group 16SrVII available in GenBank (Table 1). Analysis was performed on a sequence of the 1247 bp genomic

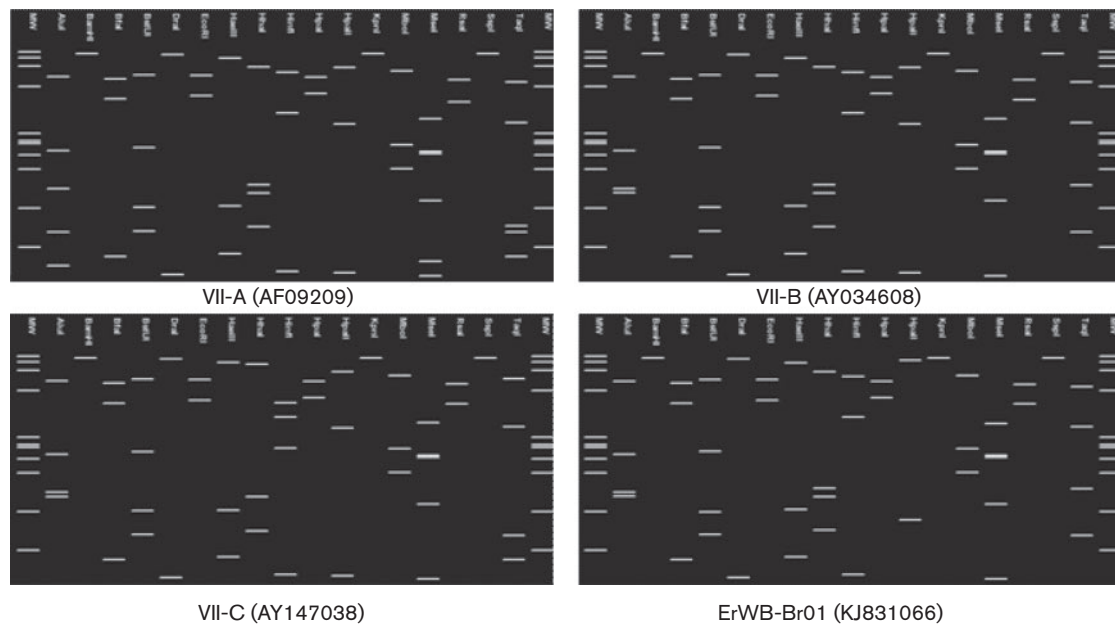
fragment of the phytoplasma strain found in erigeron. The sequences aligned and cut were exported to the restriction analysis on the virtual gel using the pDRAW32 program, developed by AcaClone Software, according to the protocol of Wei *et al.* (2007). After digestion *in silico*, an agarose gel of 3 % was plotted and captured as a separate file in PDF format for future comparisons of the profiles generated. The virtual RFLP patterns were compared and the similarity coefficient (F) was calculated for each pair of phytoplasma strains as previously described (Lee *et al.*, 1998). The phylogenetic tree was reconstructed from a sequence present in an erigeron strain (ErWB-Br01) and sequences from several phytoplasmas, using the MEGA program, with the neighbour-joining method. Bootstrapping was processed with 1000 replications and *Acholeplasma laidlawii* (M23932) was included as the outgroup.

Phytoplasma strains were detected in all symptomatic samples and the positive control, by the amplification of approximately 1.2 kb DNA fragments using the universal primer pairs. Conversely, no amplification occurred from DNA extracted from asymptomatic erigeron plants. The phytoplasma found in each symptomatic erigeron plant was considered to represent a strain and five strains were subjected to sequencing of the 16S rRNA gene. A total of three clones were sequenced per strain. These clones were compared and a majority consensus sequence was selected for each strain, since no polymorphism was observed. The selected sequences were compared and, based on the absence of polymorphism, a majority consensus sequence was chosen to represent the phytoplasma associated with the symptomatic erigeron plants. This sequence designated ErWB-Br01 (Erigeron bonariensis Witches' Broom-Brazil 01), was deposited in GenBank under accession number KJ831066.

Nucleotide sequences from EWB-Br01 demonstrated a level of similarity of 99 % when compared with the sequence of the ash yellows phytoplasma. Based on virtual RFLP patterns (Fig. 1), the similarity coefficients (F) calculated for the phytoplasma found in erigeron (EboWB phytoplasma) in relation to the other members of the 16SrVII group ranged from 0.83 to 0.94 (Table 1), indicating genetic diversity in

**Table 1.** Similarity coefficients (F) derived from the phytoplasma EboWB (16SrVII-D), identified in this study and reference phytoplasmas belonging to group 16SrVII

Phytoplasma	16S rRNA gene group-subgroup affiliation	' <i>Candidatus</i> Phytoplasma fraxini'	Erigeron witches' broom phytoplasma	Argentinian alfalfa witches' broom phytoplasma	Ebo WB phytoplasma
' <i>Candidatus</i> Phytoplasma fraxini'	16SrVII-A	1.00			
Erigeron witches' broom phytoplasma	16SrVII-B	0.90	1.00		
Argentinian alfalfa witches' broom phytoplasma	16SrVII-C	0.84	0.89	1.00	
EboWB phytoplasma	16SrVII-D	0.85	0.94	0.83	1.00



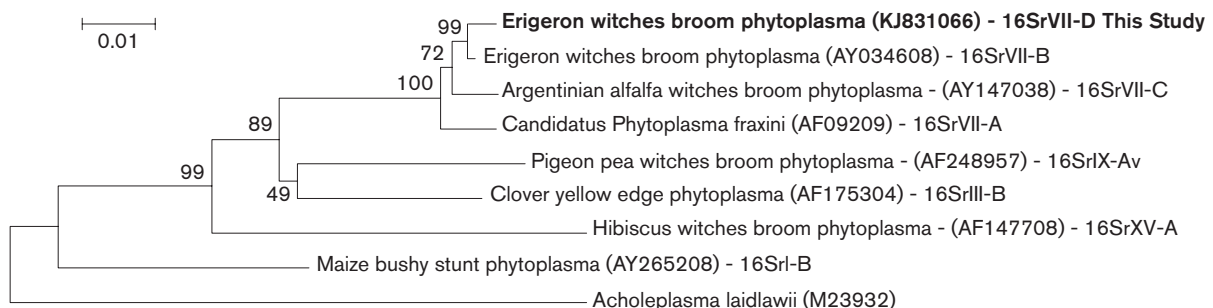
**Fig. 1.** Virtual RFLP patterns from *in silico* digestions of 16S rRNA gene R16F2n/R16R2 fragments from phytoplasma EWB-Br01 and previously known phytoplasmas belonging to distinct 16SrVII subgroups. The following restriction enzymes were used: *AluI*, *BamHI*, *BfaI*, *BstUI*, *DraI*, *EcoRI*, *HaeIII*, *HhaI*, *HinfI*, *HpaI*, *HpaII*, *KpnI*, *MboI*, *MseI*, *RsaI*, *SspI* and *TaqI*. MW,  $\phi$ X174RFHaeIII digest.

relation to the representatives of the three subgroups belonging to the 16SrVII group.

RFLP analysis of DNA fragments revealed distinct restriction patterns for the enzymes *AluI*, *HpaII*, *RsaI* and *TaqI*, when profiles derived from erigeron witches' broom phytoplasma found in this study were compared with profiles generated by the representative of the 16SrVII-A subgroup. The RFLP patterns obtained for strain EboWB were also distinct from those shown by the phytoplasma of group VII-B, but, in this case, only exhibited differences in the profile with the enzyme *HpaII*. Finally, the restriction

profiles from strain EboWB and Argentinian alfalfa witches' broom phytoplasma (16SrVII-C) were distinguishable with respect to the use of the endonucleases *HhaI*, *HinfI*, *HpaII* and *TaqI*.

The phylogenetic tree confirmed that the phytoplasma associated with erigeron witches' broom is closely related to others members of group 16SrVII. Moreover, strain EboWB emerges as a novel branch, in addition to the three other mutually distinct phytoplasmas previously classified as representatives of the subgroups within 16SrVII group (Fig. 2).



**Fig. 2.** Phylogenetic tree reconstructed using sequences of 16S rRNA of the EboWB phytoplasma associated with erigeron plants, distinct representatives of subgroups from the 16SrVII group and phytoplasmas affiliated to different groups that occur in Brazil, using the neighbour-joining method. *Achnoleplasma laidlawii* was used as the outgroup. Numbers on branches are confidence values for the bootstrap.

The presence of phytoplasmas in erigeron plants showing shoot proliferation was first reported in Brazil in the end 1970s from results obtained using an electron microscope (Kitajima & Costa, 1979). Twenty years after this discovery, now using the tools of molecular biology, a phytoplasma detected in *E. bonariensis* with witches' broom was characterized as a member of the 16SrI group (Bianchini & Bedendo, 2000). A couple of years later, a phytoplasma found in the same genus of host, which also showed symptoms of witches' broom, was described as belonging to the 16SrVII-B subgroup (Barros *et al.*, 2002). Interestingly, in the present study, a representative of a distinct subgroup was recognized in erigeron that exhibited the same type of symptoms. According to Wei *et al.* (2008), a novel subgroup is recognized if a phytoplasma strain's virtual 16S rRNA gene RFLP pattern has 0.97 or lower similarity coefficient with those of all existing representative strains of the given group. Since the subgroup 16SrVII-D phytoplasma presented values of similarity coefficients ranging from 0.83 to 0.94 when contrasted with representatives of different subgroups of group 16SrVII (Table 1), this phytoplasma may delineate a novel subgroup. Based on the virtual RFLP analysis, the endonuclease *Hpa*II was identified as the key enzyme, because it produced distinct restriction patterns that allowed distinguish the erigeron phytoplasma from representatives of other subgroups. This unique profile revealed the presence of two bands (Fig. 1), while three bands were present in the profiles exhibited by other representatives of group 16SrVII. In addition, the branching of the phylogenetic tree provided sufficient evidence to support the hypothesis that the erigeron phytoplasma represents a novel subgroup, which is more closed related to members of the 16SrVII-B subgroup, in perfect agreement with RFLP analysis that revealed only a restriction site for the enzyme *Hpa*II as a reference to distinguish these strains.

Currently, greater genetic diversity has been mainly found within three major groups (16SrI, 16SrII and 16SrIII), which, consequently, possess large numbers of subgroups (Zhao *et al.*, 2010). Conversely, the 16SrVII group consisted of only three subgroups prior to the current study. The geographical distribution of phytoplasmas belonging to this group apparently is limited to the American continent and, interestingly, representatives of the subgroup 16SrVII-A are associated with woody hosts, while members of subgroups 16SrVII-B and 16SrVII-C are present in herbaceous species (Meneguzzi *et al.*, 2008; Flôres *et al.*, 2013). The finding of the present investigation, in which the representative of the novel subgroup 16SrVII-D was identified in erigeron, follows, hence, the same pattern of the pathosystems previously reported for phytoplasmas affiliated with subgroups 16SrVII-B and 16SrVII-C. Moreover, the representative of the subgroup 16SrVII-D induced in the host similar alterations to those previously described for phytoplasmas belonging to other distinct subgroups, mainly symptoms including shoot proliferation and stunting. Thus, our findings, in addition to results

previously available, demonstrate that erigeron witches' broom may be associated with at least three distinct phytoplasmas represented by a member of group 16SrI (Bianchini & Bedendo, 2000), a member of subgroup 16SrVII-B (Barros *et al.*, 2002; Meneguzzi *et al.*, 2008) and a member of subgroup 16SrVII-D (the present study). Although erigeron plants used in this study were found in passion fruit orchards, there is no evidence that these plants can serve as reservoirs of phytoplasma for the crop, since the main agent of the witches' broom in passion fruit is a phytoplasma of the 16SrIII-B subgroup, present in various Brazilian regions (Ribeiro *et al.*, 2008).

Our findings provided firm evidence of the occurrence of a novel subgroup, expanding the knowledge about the genetic diversity within the 16SrVII group.

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