Molecular characterization of a phytoplasma affiliated with the 16SrVII group representative of the novel 16SrVII-F subgroup

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Abstract

Vernonia brasiliana is a wild perennial shrub frequently found in pasture areas. Plants of this species have been observed displaying typical symptoms induced by phytoplasmas, which were characterized by shoot proliferation, deformed leaves and leaf chlorosis. The present study confirmed the presence of phytoplasmas in association with affected plants. Sequencing of the 16S rRNA gene, computer-simulated RFLP analysis and phylogenetic analysis revealed that one of the phytoplasmas identified was representative of novel subgroup. The sequence identity scores between the novel strain and those of previously described ‘Candidatus Phylloplasma fraxini’-related strains was 99%, while similarity coefficient values were lower than 0.97. These findings provide support to delineate the phytoplasma found in vernonia plants as a reference phytoplasma for a novel subgroup designated 16SrVII-F. This representative of the novel subgroup was denominated VbSP phytoplasma (Vernonia brasiliana Shoot Proliferation; GenBank KX342018). The results of the present study revealed V. brasiliana to be a host of phytoplasmas, evidenced a novel phytoplasma associated with phytoplasmal disease in Brazil and extended the knowledge of the genetic diversity existing within the 16SrVII group.

Vernonia is a genus of the family Asteraceae with numerous species frequently present in areas of the Central Brazilian Plateau [1]. In Brazil are found approximately 180 genera of the family Asteraceae and about 900 species belonging to the genus Vernonia, whose representatives grow as herbaceous plants and shrubs [2]. Plants of Vernonia brasiliana (L.) Druce are perennial branched shrubs that can grow up to 2.5 m tall, whose branches are commonly green with abundant trichomes [3]. Plants of V. brasiliana with typical phytoplasma-induced symptoms were observed growing spontaneously in pasture fields. The symptomatic plants exhibited mainly intensive proliferation of shoots and, additionally, mild leaf chlorosis and deformed leaves.

Phytoplasmas are cell-wall-less prokaryotes, intracellular parasites that inhabit the phloem vessels and are associated with diseases of botanical species widely distributed throughout the world [4]. They are naturally vectored by sucking insects and may infect cultivated, wild and weed plants [5]. On the basis of the genetic diversity of sequences of the 16S rRNA gene, phytoplasmas are classified within groups and subgroups employing virtual RFLP patterns and similarity coefficients values [4]. The first representative of the group 16SrVII was described in the end of 1999, in the USA, in association with plants of the genera Fraxinus and Syringa [6]. This strain was classified as the reference phytoplasma for the 16SrVII-A subgroup. Later, in 2010, a similar strain was identified in peach trees cultivated in Canada [7]. Interestingly, distinct representatives of the 16SrVII group have been characterized only in countries of South America [8]. Thus, in Brazil, a strain affiliated with the 16SrVII-B subgroup was delineated from naturally infected plants of Erigeron sp. and Catharanthus roseus [9]. A subgroup 16SrVII-B phytoplasma was also identified in erigeron plants sampled in fields located in Argentina [10]. In this same country, a genetic variant within the 16SrVII group was found in areas seeded with alfalfa, which was described as the reference phytoplasma for the 16SrVII-C subgroup [11]. In Brazil, a 16SrVII-C phytoplasma was identified in legumes known as sunn hemp displaying shoot proliferation [12]. In addition, the occurrence of one more distinct strain of this group, which was identified in erigeron plants and designed as representative of the 16SrVII-D subgroup has been demonstrated [13]. Recently, a phytoplasma associated with Chilean grapevine yellows, previously classified as belonging to subgroup 16SrVII-A [8], was reclassified as a representative of subgroup 16Sr-E [14].
Based on the results of the present investigation we report *Vernonia brasiliana* as a new host for phytoplasmas in Brazil and delineate a novel phytoplasma as representative of the novel 16SrVII-F subgroup.

Samples comprising symptomatic and asymptomatic plants were collected from pasture fields located in three States (Minas Gerais, Paraná and São Paulo), situated in the southeast region of Brazil. Extraction of total DNA was performed from fresh foliar tissue, using the method described by James and collaborators [15]. Universal primer pairs that amplify the 16S rRNA gene were employed in nested PCR assays for detection of phytoplasmas. PCR products generated in the first reaction by the primers R16mF2/R16mR1 [16] were used as templates in the second reaction primed by R16F2n/R16R2 [16]. Positive and negative controls were represented by DNA extracted from infected periwinkle (*Catharanthus roseus*) and asymptomatic vernonia plants, respectively. The DNA fragments of 1.2 kb amplified by nested PCR were ligated into the pGEM T Easy Vector System I (Promega), transformed into *Escherichia coli* DH5α, and subsequently sequenced using the primers pair SP6/T7 [17]. The sequences were aligned, compared among themselves, with the sequences belonging to phytoplasmas of distinct groups and with sequences of phytoplasmas representative of different subgroups within group 16SrVII. The sequences were analyzed using computer programs for construction and sequence analysis such as Bioedit, V.7.0.9.0 [18] and Multiple Sequence Alignment — CLUSTALW.

Computer-simulated RFLP analysis was conducted using a consensus sequence of the phytoplasma found in vernonia and sequences of reference phytoplasmas of all subgroups of the 16SrVII group available in the GenBank (Table 1). Subsequently, in silico restriction analysis and virtual RFLP plotting was performed using the pDRAW32 program, developed by AcaciaSoftware [4]. All sequences were digested with a set of 17 restriction enzymes as recommended previously [4, 19]. The virtual RFLP patterns were compared and similarity coefficient (F) values were calculated for each pair of phytoplasma strains as proposed by Lee and collaborators [20]. The sequence representing the phytoplasma associated with vernonia and sequences from phytoplasmas affiliated with distinct subgroups of the 16SrVII group were used in a phylogenetic tree reconstructed by using MEGA program [21] with the neighbour-joining method. Electron microscopy was performed with small segments of leaf veins, appropriately prepared according to methodology previously described [22, 23].

The presence of phytoplasmas was revealed in 82 and 30 % of the symptomatic and asymptomatic vernonia plants, respectively, by the amplification DNA fragments of 1.2 kb generated by nested PCR. Molecular detection was confirmed by transmission and scanning electron microscopy that evidenced the occurrence of phytoplasmas by visualization of pleomorphic and round bodies in the phloem vessels of positive samples (Fig. 1). The phytoplasma detected in each positive plant (sample) was denominated as a strain and 30 % of the strains found in these positive plants were submitted to sequencing of the 16S rRNA. For each strain three clones were sequenced, which were compared among themselves, and a majority consensus sequence was selected as representative of each strain. The sequencing revealed that approximately 8 % of the strains belonged to the 16SrVII group. The nucleotide sequence named VbSP-Br29 (*Vernonia brasiliana* Shoot Proliferation-Brazil 29) was chosen to represent the strain of the 16SrVII group present in vernonia plants and it was deposited in GenBank under the accession number (KX342018).

Analysis of the sequence VbSP-Br29 evidenced 99 % similarity with sequences representative of the reference phytoplasmas of different subgroups within 16SrVII group. Similarity coefficients (F) values based on virtual RFLP patterns ranged from 0.60 to 0.96 when the phytoplasma associated with vernonia was contrasted with phytoplasmas affiliated with distinct 16SrVII subgroups (Table 1). The collective virtual RFLP profiles allowed the phytoplasma found in vernonia to be distinguished from all the reference phytoplasmas of the different subgroups within the 16SrVII group (Fig. 2). More specifically, the identified phytoplasma was distinguished from the subgroup 16SrVII-A strain (*Candidatus* Phytoplasma fraxini - AF092209) with the enzymes *AluI*, *HinfI*, *MseI*, and *TaqI*. However, this

**Table 1.** Similarity coefficients (F) calculated from analysis of virtual RFLP patterns from phytoplasmas affiliated with distinct subgroups of the group 16SrVII and the vernonia phytoplasma (VrSP-Br29) representative of the novel subgroup 16SrVII-F.

<table>
<thead>
<tr>
<th>Phytoplasmas</th>
<th>Subgroups of 16SrVII</th>
<th>AshY1</th>
<th>ErlWB5</th>
<th>ArAWB</th>
<th>ErWB-Bro01</th>
<th>CGyP</th>
<th>VbSP-Br29</th>
</tr>
</thead>
<tbody>
<tr>
<td>'Candidatus Phytoplasma fraxini'</td>
<td>16SrVII-A</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Erigeron witches'-broom</td>
<td>16SrVII-B</td>
<td>0.92</td>
<td>1</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Argentinian alfalfa witches'-broom</td>
<td>16SrVII-C</td>
<td>0.86</td>
<td>0.89</td>
<td>1</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Erigeron bonariensis witches'-broom</td>
<td>16SrVII-D</td>
<td>0.87</td>
<td>0.94</td>
<td>0.83</td>
<td>1</td>
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<td></td>
</tr>
<tr>
<td>Chilean grapevine yellows</td>
<td>16SrVII-E</td>
<td>0.64</td>
<td>0.57</td>
<td>0.55</td>
<td>0.62</td>
<td>1</td>
<td></td>
</tr>
<tr>
<td>Vernonia shoot proliferation</td>
<td>16SrVII-F</td>
<td>0.91</td>
<td>0.96</td>
<td>0.88</td>
<td>0.80</td>
<td>0.60</td>
<td>1</td>
</tr>
</tbody>
</table>

AshY1 (‘Candidatus Phytoplasma fraxini’-AF092209); ErlWB5 (Erigeron witches’ broom-AY034608); ArAWB (Argentinian alfalfa witches’ broom-AY147038); ErWB-Bro01 (Erigeron bonariensis witches’ broom-Bro01-KJ831066); CGyP (Chilean grapevine yellows- AY741531); VbSP-Br29 (*Vernonia brasiliana* Shoot Proliferation-KX342018).
Phytoplasma was divergent from the 16SrVII-B strain (Erigeron witches’-broom phytoplasma – AY034608) only for the endonuclease HinfI. The enzymes HhaI, HinfI and TaqI permitted separation of the vernonia phytoplasma from the strain of the 16SrVII-C subgroup (Argentinian alfalfa witches’-broom phytoplasma – AY147038). The studied phytoplasma can be differentiated from the representative of the 16SrVII-D subgroup (Erigeron witches’-broom phytoplasma – KJ831066) by the restriction patterns derived with the enzymes HinfI and HpaII. Finally, vernonia phytoplasma was different from representative of subgroup 16SrVII-E (Chilean grapevine yellows phytoplasma – AY 741531) in relation to the enzymes AluI, DraI, Hhal, HinfI, MseI and TaqI.

The phylogenetic tree, reconstructed with sequences from phytoplasmas affiliated with distinct subgroups of the 16SrVII group and the vernonia shoot proliferation phytoplasma, revealed that the latter (Fig. 3) gave rise to a distinct branch, indicating the occurrence of a novel subgroup component of the 16SrVII group.

The initial suspicion of phytoplasmal disease based on symptoms was confirmed consistently by the high occurrence of association between phytoplasmas and affected vernonia plants. Although phytoplasmas have been detected more frequently in symptomatic samples, symptomless plants were also found as harbouring of these prokaryotes. Among the different phytoplasmas identified in association with vernonia, it was discovered a strain distinct from those reference strains representing components of the 16SrVII group. Virtual RFLP analysis and values of similarity coefficients (F) provided support for delineation of a novel subgroup 16Sr phytoplasma, based on the scheme proposed by Wei and collaborators [4]. According to these authors a novel subgroup is recognized when a strain presents a F value equal to or lower than 0.97 in comparison to those of all of the existing representative strains of a specific group. The strain identified in this study showed F values equal or lower than 0.96 in relation to the representatives of the subgroups hitherto known within the 16SrVII group (Table 1). Therefore, this phytoplasma represents a distinct subgroup, 16SrVII-F. The endonuclease HinfI is the key enzyme that produced restriction patterns that made it possible to distinguish the vernonia phytoplasma from other 16SrVII phytoplasmas (Fig. 2). These findings are also supported by the tree generated from phylogenetic analysis that evidenced vernonia phytoplasma to be affiliated with monophyletic group of phytoplasmas making up group 16SrVII. More specifically, the vernonia phytoplasma represents a distinct emergent branch that constitutes the novel 16SrVII-F subgroup.

**Fig. 1.** Pleomorphic bodies of phytoplasma found in the phloem vessels of vernonia plants by scanning electron microscopy (top row) and transmission electron microscopy (bottom row).
Interestingly the results of molecular characterization revealed that representatives of the subgroups 16SrVII-B [9], 16SrVII-D [13] and 16SrVII-F (this study) registered in Brazil were more closely related in comparison with phytoplasmas belonging to groups 16SrVII-A described in the USA [6, 8], 16SrVII-C reported in Argentina [11], and

![Image](https://example.com/image.png)

**Fig. 2.** Virtual RFLP patterns generated by the sequences of the 16S rRNA gene from phytoplasma belonging to distinct 16SrVII subgroups including vernonia phytoplasma (VbSP phytoplasma) delineated as the novel 16SrVII-F subgroup. The following restriction enzymes were used: Alul, BamHI, Bfai, BstUI, DraI, EcoRI, HaeIII, HhaI, Hinfl, Hpal, HpaII, KpnI, Mbol, MseI, RsaI, SspI, TaqI. Molecular weight marker, φX174-DNA HaeIII digest.

![Image](https://example.com/image.png)

**Fig. 3.** Phylogenetic tree based on 16S rRNA gene sequences from reference phytoplasmas of subgroups within the 16SrVII group and the phytoplasma associated with vernonia shoot proliferation (VbSP phytoplasma) using the neighbour-joining method. Bootstrap was processed 1000 times and *Acholeplasma laidlawii* ATCC 23206 was included as an outgroup. Numbers on branches indicate confidence values for the bootstrap.
16SrVII-E identified in Chile [8, 14]. These findings were according to both similarity coefficients calculation (Table 1) and evolutionary evidence derived from phylogenetic analysis (Fig. 3). These results are also in agreement with those of virtual RFLP analysis, which revealed that only one restriction enzyme distinguishes the member of subgroup 16SrVII-D from the member of 16SrVII-B [13] and one restriction enzyme distinguishes the reference strain of subgroup 16SrVII-F from representatives of the subgroups 16SrVII-B and 16SrVII-D (this study).

A small number of representatives have been described for the 16SrVII group involving five distinct strains examined in works published prior to the present study. Thus, the present investigation contributes one more subgroup to those currently known. Moreover a few species have been found as hosts of phytoplasmas of group 16SrVII, which are represented by ash [6], common lilac [6], peach [7], grapevine [8, 14], species of the genus Erigeron [9, 10, 13], periwinkle [9], alfalfa [11] and sunn hemp [12]. On the basis of the results obtained in this study, V. brasiliana can also be included as a novel host for phytoplasma of the 16SrVII group.

In the last two decades, although numerous novel groups and subgroups of phytoplasmas have been reported in diverse regions of the world [24], representatives of the 16SrVII group have been described only in some countries of North and South America [13]. The present work, hence, in addition to papers previously published, contributes with the identification of a novel strain affiliated with this small group of phytoplasmas, whose members are apparently restricted to the American continent. The discovery of the 16SrVII-F subgroup, which is reported by first time, to our group of phytoplasmas, whose members are apparently 16SrVII group have been described only in some countries diverse regions of the world [24], representatives of the group.


Conflicts of interest
The authors declare that there is no conflict of interest.

References