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Molecular characterization of a phytoplasma of group 16SrIX related to '*Ca.* Phytoplasma phoenicium' in periwinkle in Brazil

Júlio César Barbosa[1], Bárbara Eckstein[2], Armando Bergamin Filho[1], Ivan Paulo Bedendo[1] & Elliot W. Kitajima[1]

[1]Departamento de Entomologia, Fitopatologia e Zoologia Agrícola, Universidade de São Paulo, ESALQ, 13418-900, Piracicaba, SP, Brazil; [2]Departamento de Genética, Universidade Federal do Paraná, 81531-990, Curitiba, PR, Brazil

Author for correspondence: Júlio C. Barbosa, e-mail: juliobarbosao@gmail.com

ABSTRACT

Periwinkle (*Catharanthus roseus*), a tropical perennial plant, was found to be infected by a phytoplasma. Plants exhibiting virescence, phyllody and variegation symptoms were collected in the states of Minas Gerais and São Paulo, Brazil. The phytoplasma was transmitted by grafting from an infected periwinkle plant to healthy plants and by dodder to a citrus plant. Phytoplasma isolates from periwinkle plants from Brazil had the 16S rDNA gene sequenced and were classified in the 16SrIX group, subgroup A, belonging to the '*Candidatus* P. phoenicium' species.
Key words*:* *Catharanthus*, 16S rRNA, mollicutes.

RESUMO

Caracterização molecular de um fitoplasma do grupo 16SrIX relacionado ao '*Ca.* Phytoplasma phoenicium' em vinca no Brasil

Fitoplasmas foram encontrados em vinca (*Catharanthus roseus*), uma planta tropical herbácea. Plantas exibindo sintomas de virescência, filodia e variegação foram coletadas nos estados de Minas Gerais e São Paulo. O fitoplasma foi transmitido por enxertia a partir de uma planta de vinca infectada para plantas sadias da mesma espécie, e por *Cuscuta* sp. para uma planta sadia de citrus. Isolados de fitoplasma encontrados em plantas de vinca no Brasil tiveram a região 16S rDNA sequenciada e foram classificados no grupo 16SrIX, subgrupo A, pertencente a espécie '*Candidatus* P. phoenicium'.
Palavras-chave*:* *Catharanthus*, mollicutes, RNA 16S.

Phytoplasmas are prokariotes of the class Mollicutes which infect more than 700 plant species around the world (Bertaccini et al., 2007) and are vectored by phloem-feeding leafhoppers, planthoppers and psyllids (Weintraub & Beanland, 2006). They have still not been cultivated *in vitro*, therefore, molecular methods are the best approach for their detection, identification and classification. Based on nucleotide sequence similarity of the 16S rRNA gene, phytoplasmas are classified as '*Candidatus*' species (IRPCM, 2004). In parallel, phytoplasmas are also classified into groups and subgroups using restriction fragment length polymorphism (RFLP) pattern similarities based on seventeen restriction enzymes (Lee et al., 1998; Zhao et al., 2009).

Periwinkle [*Catharanthus roseus* (L.) G. Don] is a tropical perennial plant belonging to the *Apocyanaceae* family used mainly as an ornamental plant. This plant contains a wide range of monoterpenoid indole alkaloids that may be used in the treatment of hypertension and in cancer chemotherapy (Leménager et al., 2005). In addition, periwinkle plants have been known in plant pathology as model host plant to study phytoplasma-plant interactions (De Luca et al., 2010, Chen & Lin, 2011) and where phytoplasma strain collections are maintained (Favali et al., 2008). The natural infection of periwinkle plants by phytoplasmas belonging to the 16SrII, 16SrIII, 16SrIX, and 16SrXV groups has been reported in Brazil (Barros et al., 1998, Bedendo et al., 1999, Montano et al., 2001a, 2001b). Phytoplasmas of the 16SrIX group were found in these plants at some states of Brazil including Rio Grande do Norte (RN), Pernambuco (PE), São Paulo (SP) (Barros et al., 1998) and Mato Grosso (MT) (Bedendo et al., 1999). However, a complete classification of these phytoplasmas in Brazilian periwinkle plants has not yet been carried out.

In July 2009 and January 2010, periwinkle plants exhibiting virescence, phyllody and variegation symptoms (Figure 1) suggesting a phytoplasma infection were found in public gardens in the municipalities of Carmo do Paranaíba, state of Minas Gerais and Araraquara, state of São Paulo, Brazil. Three symptomatic periwinkle samples were collected at each place and further analyzed for the presence of phytoplasmas.

Total DNA was separately extracted from each sample according to Dellaporta et al. (1983). PCR amplifications were performed using the universal phytoplasma primers P1/P7 (Schneider et al., 1995) followed by primers R16F2n/

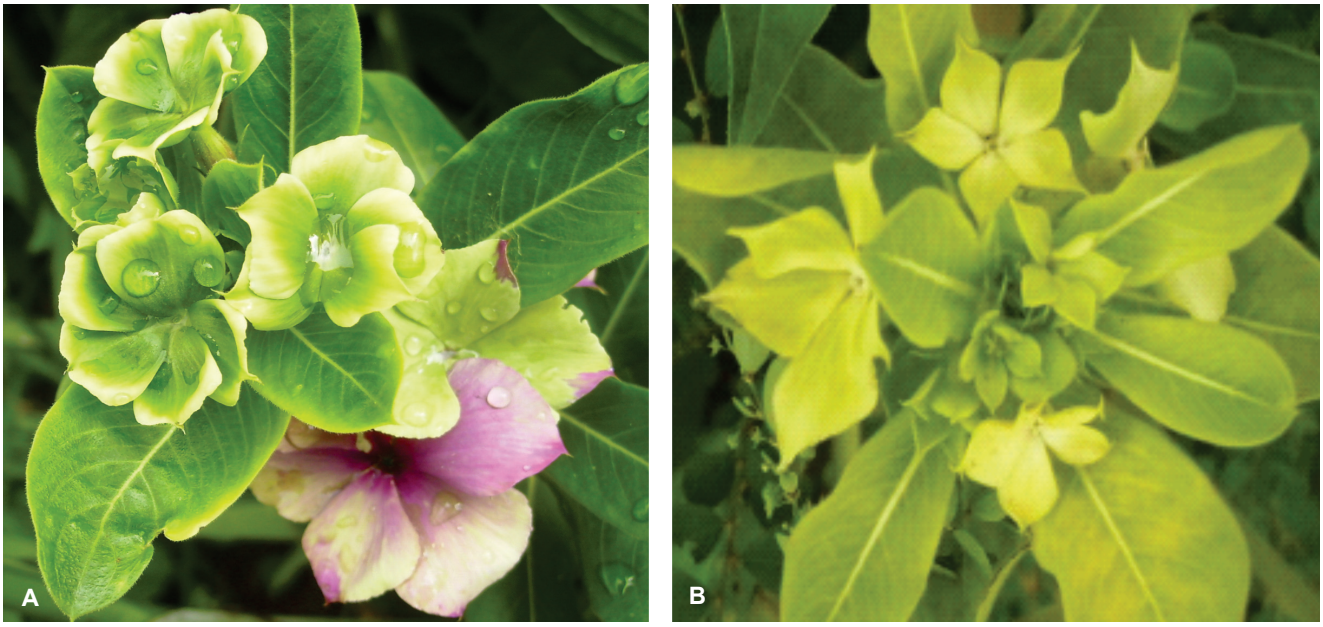

FIGURE 1 - Virescence (A and B) and variegation (A) symptoms in periwinkle plants. **A.** Sample collected in Araraquara, São Paulo state; **B**. Sample collected in Carmo do Paranaíba, Minas Gerais state.

R16R2 (Gundersen & Lee, 1996). In all analyzed samples we observed fragments of approximately 1.25 kb, indicating the infection by phytoplasma. The phytoplasma strains from periwinkle plants samples collected in Araraquara and Carmo do Paranaíba were named PwK-AR1 to PwK-AR3 and PwK-CP1 to PwK-CP3, respectively. PCR amplified products were separately analyzed by restriction endonuclease digestion with the following enzymes: *Alu* I, *Hae* III, *Hha* I, *Hinf* I, *Rsa* I and *Taq* I. The restriction products were then separated by electrophoresis in a 5% polyacrylamide gel and stained with Sybr Safe (Invitrogen). DNA bands were visualized with a UV transiluminator.

RFLP patterns produced were similar to those characteristic of strains belonging to the group 16SrIX (Lee et al., 1998) (Figure 2), confirming that this phytoplasma is widely disseminated in Brazil and has been maintained in periwinkle plants, since its presence was already described in many Brazilian states more than ten years ago (Barros et al., 1998; Bedendo et al., 1999). However, as the characterization of those phytoplasmas was based only on RFLP analysis with a limited number of restriction enzymes, it is not possible to know if all of them are really related.

To better characterize and classify the phytoplasmas of the 16SrIX group infecting periwinkle plants in Brazil, PCR amplified products of PwK-AR1 and PwK-CP3 strains from Araraquara and Carmo do Paranaíba, respectively, were cloned in the pGEM-T vector (Promega) and transformed into *Escherichia coli* DH5α according to standard procedures (Sambrook et al., 1989). Two clones from each sample were sequenced in both orientations. The 16S rRNA sequences of PwK-AR1 and PwK-CP3 strains share 100% identity. Thus, the consensus 16S rRNA sequence (1250 pb) of PwK-AR1(GenBank accession: JN792515) and PwK-CP3 (GenBank accession: JN792516) strains were subjected to virtual RFLP analysis using the pDRAW32 (AcaClode Software) and *i*PhyClassifier programs (Zhao et al., 2009) for RFLP pattern comparisons and calculation of the similarity coefficient, according to Zhao et al. (2009). Sequence analysis and comparisons were performed using the BLAST analysis (Altschul et al., 1997). A phylogenetic tree of 16S rDNA sequences was constructed using MEGA 4.0 (Tamura et al., 2007) after multiple alignments obtained with Clustal W (Thompson et al., 1994).

The collective virtual RFLP pattern of the PwK-AR1 and PwK-CP3 16S rDNA sequences (Figure 3) was similar to reference patterns of group 16SrIX phytoplasmas. This finding confirms the results obtained by RFLP/gel electrophoretic analysis (Figure 2). The RFLP pattern similarity coefficients of 1.0 between the phytoplasmas of this work and the reference pattern of group 16Sr IX, subgroup A (GenBank accession: AF248957) indicates that PwK-AR1 and PwK-CP3 strains are members of 16SrIX A subgroup A. The 16S rDNA sequence analyses showed that PwK-AR1 and PwK-CP3 strains shared 99% of similarity with '*Ca.* Phytoplasma phoenicium' strains, including the HLB-associated phytoplasma (GenBank accession: HQ423159) which has been associated to huanglongbing symptoms in São Paulo state, Brazil (Teixeira et al., 2008). The IRPCM Phytoplasma/Spiroplasma Working Team-Phytoplasma Taxonomy Group has suggested that

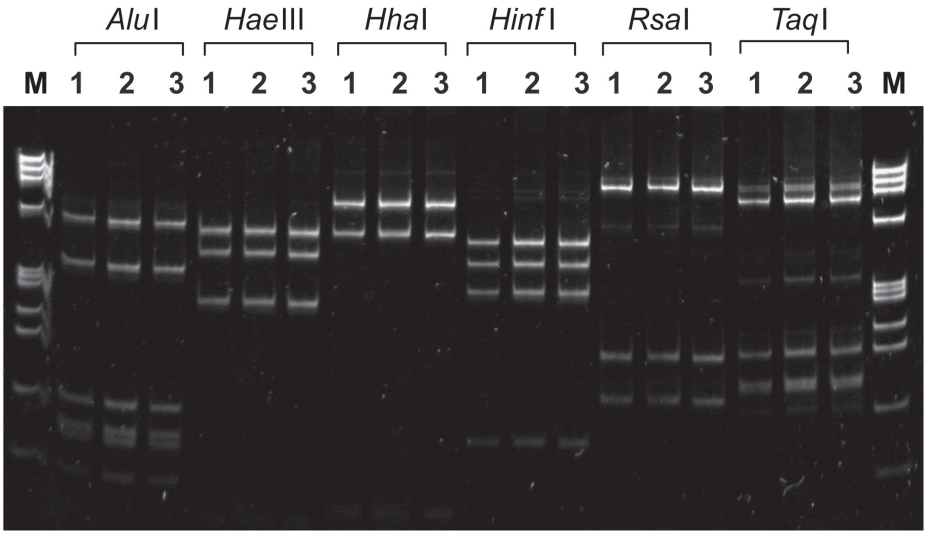

FIGURE 2 - RFLP analyses of 16S rDNA fragments (1.25 Kb) from representative PwK-AR1, PwK-AR2, and PwK-CP3 strains digested with *Alu*I, *Hae*III, *Hha*I, *Hinf*I, *Rsa*I, and *Taq*I restriction enzymes. Lanes: 1, PwK-AR1 strain; 2, PwK-AR2 strain; 3, PwK-CP3 strain; M, ΦX174 DNA, HaeIII digest.

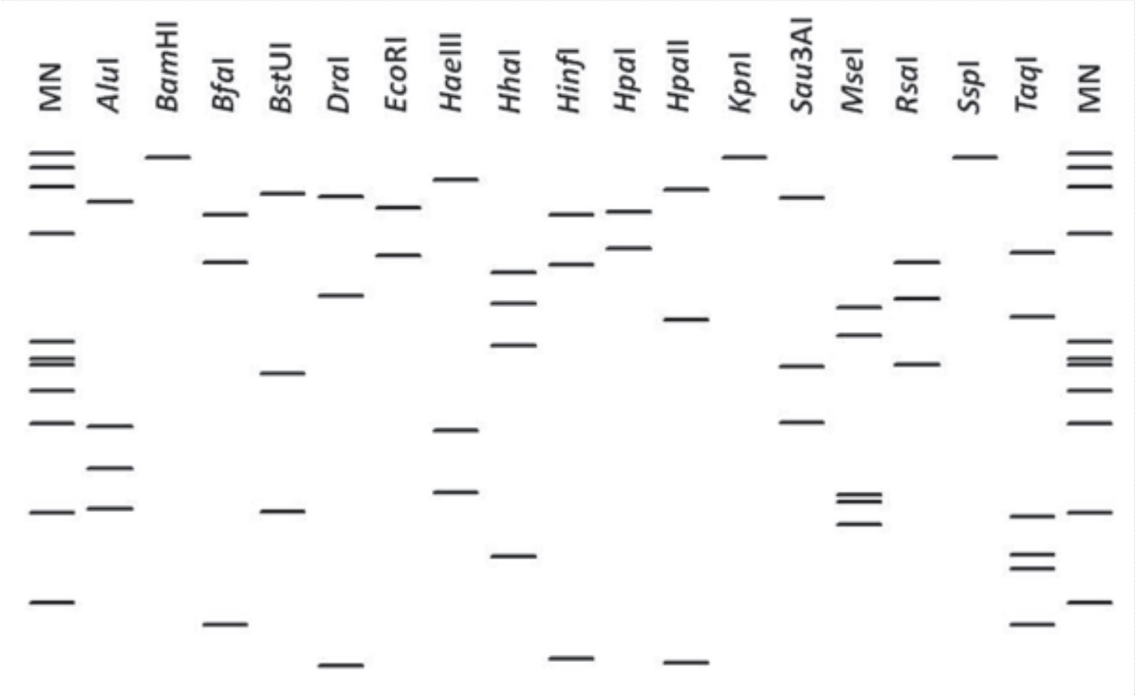

FIGURE 3 - Virtual RFLP patterns derived from *in silico* digestions of 16S rDNA (1.25 kbp fragments) from the PwK-CP3 strain using 17 restriction endonuclease enzymes: *Alu*I, *Bam*HI, *Bfa*I, *Bst*UI, *Dra*I, *Eco*RI, *Hae*III, *Hha*I, *Hinf*I, *Hpa*I, *Hpa*II, *Kpn*I, *Sau*3AI, *Mse*I, *Rsa*I, *Ssp*I, and *Taq*I. MW, ΦX174 DNA, *Hae*III digest.

phytoplasma whose 16S rRNA nucleotides sequences exceed 97.5% similarity should be considered strains of the same '*Ca*. Phytoplasma' species (IRPCM 2004). Following this guideline, phytoplasmas of the present study belong to '*Ca*. Phytoplasma phoenicium'.

In the tree based on 16S rDNA sequences (Figure 4), PwK-AR1 and PwK-CP3 strains are located in a monophyletic branch with 100% bootstrap value including the HLB-associated phytoplasma and other representative phytoplasmas of group 16Sr IX, subgroups B, C, D and E which also belong to the '*Ca*. P. phoenicium' specie. These results reinforce those obtained with sequence comparisons.

The PwK-AR1 strain was transmitted by graft from an infected periwinkle plant to healthy periwinkle plants. Grafted periwinkle plants showed leaf yellowing symptoms (Figure 5). This strain was also transmitted by dodder (*Cuscuta campestris* Yunck) from the grafted

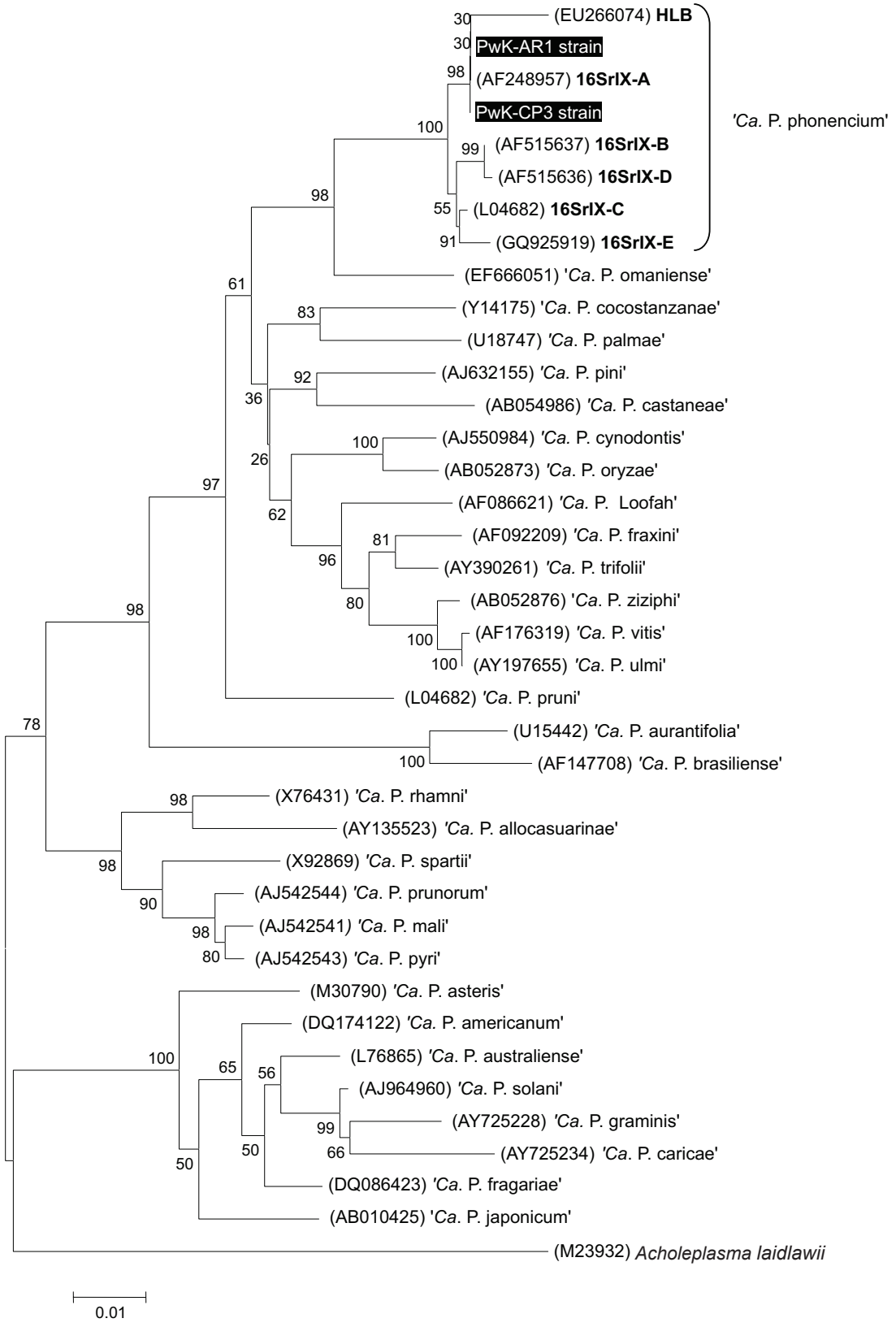

FIGURE 4 - Phylogenetic tree inferred from 16S rDNA sequences the using Neighbour-Joining method with 1000 bootstrap replications and default parameters. The numbers at the nodes indicate the percent bootstrap values. The scale indicates the number of substitutions per site. The taxa used in the phylogenetic tree construction included reference strains belonging to '*Candidatus*' species previously described according to the IRPCM Phytoplasma/Spiroplasma Working Team - Phytoplasma Taxonomy Group (IRPCM, 2004). *Acholeplasma laidlawii* was used as an outgroup.

FIGURE 5 - Leaf yellowing symptoms in citrus **A.** and periwinkle **B.** following dodder and graft transmission, respectively, from an infected periwinkle plant.

periwinkle plant to a *Citrus limonia* Osbesck plant, which also exhibited leaf yellowing symptoms (Figure 5). The presence of phytoplasma into transmitted periwinkle and citrus plants was confirmed by nested PCR assay employing primers P1/P7 and R16F2n/R16R2 as described previously (*data not shown*). The symptoms observed in citrus plant are in part similar to those displayed in citrus plants infected by the HLB-associated phytoplasma (Teixeira et al., 2008). According to the same authors, the HLB-associated phytoplasma is probably transmitted to citrus from an external source of inoculum which could be weeds, crop plants or even ornamental plants as periwinkle.

This is the first time that phytoplasmas of the 16SrIX group from periwinkle plants are sequenced and classified at the subgroup level in Brazil, contributing to the increase of the knowledge of phytoplasma diversity in tropical areas and also indicating that periwinkle may serve as a natural reservoir for this pathogen.

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