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‘*Candidatus* Phytoplasma sudamericanum’, a novel taxon, and strain PassWB-Br4, a new subgroup 16SrIII-V phytoplasma, from diseased passion fruit (*Passiflora edulis* f. *flavicarpa* Deg.)

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Symptoms of abnormal proliferation of shoots resulting in formation of witches’-broom growths were observed on diseased plants of passion fruit (*Passiflora edulis* f. *flavicarpa* Deg.) in Brazil. RFLP analysis of 16S rRNA gene sequences amplified in PCRs containing template DNAs extracted from diseased plants collected in Bonito (Pernambuco) and Viçosa (Minas Gerais) Brazil, indicated that such symptoms were associated with infections by two mutually distinct phytoplasmas. One phytoplasma, PassWB-Br4 from Bonito, represents a new subgroup, 16SrIII-V, in the X-disease phytoplasma group (‘*Candidatus* Phytoplasma pruni’-related strains). The second phytoplasma, PassWB-Br3 from Viçosa, represents a previously undescribed subgroup in group 16SrVI. Phylogenetic analyses of 16S rRNA gene sequences were consistent with the hypothesis that strain PassWB-Br3 is distinct from previously described ‘*Ca.* Phytoplasma’ species. Nucleotide sequence alignments revealed that strain PassWB-Br3 shared less than 97.5 % 16S rRNA gene sequence similarity with previously described ‘*Ca.* Phytoplasma’ species. The unique properties of its DNA, in addition to natural host and geographical occurrence, support the recognition of strain PassWB-Br3 as a representative of a novel taxon, ‘*Candidatus* Phytoplasma sudamericanum’.

Bacteria in the class *Mollicutes* include an increasingly large group known foremost as plant pathogens, the phytoplasmas (formerly mycoplasma-like organisms, MLOs). In plants, phytoplasmas reside in sieve cells of phloem tissue and induce diseases often characterized by symptoms that are indicative of disturbances in the balance of plant growth regulators. The AT-rich genomes of phytoplasmas are relatively small in size (Marcone *et al.*, 1999), a feature that is attributable, at least in part, to evolutionary loss of diverse genes that have become unnecessary in an ongoing process of adaptations enabling import of critical nutrients from hosts (Davis *et al.*, 2005). The evolutionary genome reduction probably accounts for the inability to obtain pure cultures of phytoplasmas *in vitro*.

In spite of the inability to obtain phytoplasmas in pure culture, advances in DNA-based technologies over the past two decades have enabled rapid and sensitive detection of phytoplasmas in infected hosts and have provided the basis for establishment of a provisional ‘*Candidatus* species’ taxonomy (Gundersen *et al.*, 1994; IRPCM Phytoplasma/Spiroplasma Working Team – Phytoplasma Taxonomy Group, 2004). Thus far, 30 ‘*Candidatus* Phytoplasma’ taxa have been formally described on the basis of 16S rRNA gene sequence analyses (Lee *et al.*, 2011; Malembic-Maher *et al.*, 2011; Zhao *et al.*, 2009a). Progress has yielded the characterization and phylogenetic analysis of numerous phytoplasma strains, expansion of the phytoplasma 16S rRNA gene RFLP group/subgroup classification system, construction of a web-based interactive site (*i*PhyClassifier,

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Abbreviations: PassWB, passion fruit witches’-broom; PFP, passion fruit proliferation.

The GenBank/EMBL/DDBJ accession numbers for the 16S rRNA gene sequences of strains PassWB-Br3 and PassWB-Br4 are GU292081 and GU292082, respectively.

http://plantpathology.ba.ars.usda.gov/cgi-bin/resource/iphyclassifier.cgi) for phytoplasma identification and classification, identification of gene sequences useful for differentiating closely related phytoplasmas, and the delineation of at least an additional 15 potentially new '*Candidatus* Phytoplasma' taxa (IRPCM Phytoplasma/Spiroplasma Working Team – Phytoplasma Taxonomy Group, 2004; Wei *et al.*, 2007; Zhao *et al.*, 2009b).

In this communication, we report results from the study of phytoplasmas associated with disease of passion fruit in Brazil. Passion fruit (*Passiflora edulis* f. *flavicarpa* Deg., family *Passifloraceae*), also known as yellow passion fruit, is an important agricultural crop in tropical and subtropical regions of the world. The plant is widely cultivated commercially in Brazil, where it is recognized as a flavourful source of vitamins and is used in diverse food products and beverages (Aguiar-Menezes *et al.*, 2002). Diseases of passion fruit possibly associated with plant infections by phytoplasmas have been reported previously (Kitajima 1994; Kitajima *et al.*, 1978, 1981, 1986). Passion fruit witches'-broom (PassWB) disease was first reported in the states of Rio and Pernambuco, Brazil, by Kitajima *et al.* (1981). Symptoms of the disease in both locations consisted of abnormal proliferation of axillary shoots (Kitajima *et al.*, 1981; Loreto & Vital, 1983). Though not frequent, PassWB caused severe losses where it occurred. Through the use of electron microscopy, a phytoplasma was subsequently found to be associated with witches'-broom disease (Chagas & Moraes de Oliveira, 1987; Costa *et al.*, 1993; Kitajima & Chagas, 1984). Although the association of phytoplasmas with disease(s) of passion fruit has been recognized for over 30 years, little work has been done to characterize the phytoplasma(s) (Davis *et al.*, 1994; Ribeiro, 2008; Ribeiro *et al.*, 2008).

In the present study, diseased plants of passion fruit exhibiting symptoms of abnormal proliferation of axillary shoots and witches'-broom growths were observed in the states of Pernambuco (PE) and Minas Gerais (MG), Brazil. Work was initiated to determine the possible association of phytoplasma(s) with PassWB disease in these regions and to characterize the associated phytoplasmas. Samples of symptomatic shoot tissue were collected from naturally infected passion fruit plants (PassWB-Br3 and PassWB-Br4) growing in Viçosa, MG, and Bonito, PE, respectively. Nucleic acid was extracted from each sample and used as a template in separate, nested PCRs primed by primer pairs P1/P7 and 16SF2n/16SR2n (F2n/R2n) as described previously (Gundersen & Lee, 1996). Amplification of phytoplasma-characteristic DNA fragments in the PCRs indicated that the plants were possibly infected with phytoplasmas. The 1.2 kb rRNA gene products of PCRs primed by F2n/R2 were analysed by single enzyme digestion with *Alu*I, *Bfa*I, *Dra*I, *Eco*RI, *Hae*III, *Hha*I, *Hin*fI, *Hpa*I, *Hpa*II, *Kpn*I, *Sau*3AI, *Mse*I, *Rsa*I, and *Taq*I (New England Biolabs) according to the manufacturer's instructions. Results from comparative analysis of collective RFLP patterns of the 1.2 kb 16S rRNA gene segments indicated that the two passion fruit plants were infected by different phytoplasmas. These collective RFLP patterns were in complete agreement with results from subsequent virtual RFLP analyses using *i*PhyClassifier. RFLP patterns of the 1.2 kb rRNA gene segment amplified from PassWB-Br4

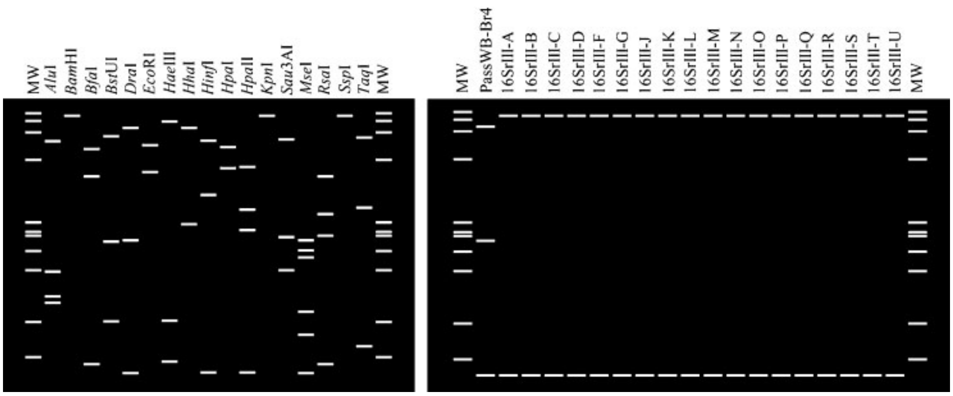

Fig. 1. Virtual RFLP patterns derived from *in silico* digestions, using *i*PhyClassifier, of 16S rRNA gene F2n/R2 fragments from PassWB phytoplasma strain PassWB-Br4 using 17 restriction endonuclease enzymes (left): *Alu*I, *Bam*HI, *Bfa*I, *Bst*UI, *Dra*I, *Eco*RI, *Hae*III, *Hha*I, *Hin*fI, *Hpa*I, *Hpa*II, *Kpn*I, *Sau*3AI, *Mse*I, *Rsa*I, *Ssp*I and *Taq*I. Virtual *Dra*I (right) RFLP patterns distinguishing strain PassWB-Br4 from other strains in group 16SrIII. The subgroups are represented by the following strains (accession nos and strain designations are in parentheses): subgroup III-A (L33733, strain CX), III-B (AF189288, CYE-Or), III-C (FJ376626, PB1), III-D (FJ376627, GR1), III-F (AF510724, MW1), III-G (AF190226, WWB), III-J [AF147706; ChWBIII (Ch10)], III-K (AF274876, SLF), III-L (EU169138, EF-MM), III-M (FJ226074, PPT-MT117-1), III-N (FJ376629, PPT-AK6), III-O (AF370120, DanVir *rrnB*), III-P (AF370120, DanVir *rrnA*), III-Q (AF302841, BRWB), III-R (AF373105, CirWL), III-S (L04682, WX), III-T (FJ231728, ChDT17), and III-U (HM589213, EB02-Br06). The restriction fragments were resolved by *in silico* electrophoresis through 3 % agarose gel. MW, ΦX174 DNA–*Hae*III digest.

were similar to patterns characteristic of strains classified in group 16SrIII. RFLP patterns of the 1.2 kb rRNA gene segment amplified from PassWB-Br3 exhibited similarities with those characteristic of strains in two groups, 16SrVI (clover proliferation phytoplasma group) and 16SrVII (ash yellows phytoplasma group).

The rRNA gene products amplified in PCRs were sequenced by automated DNA sequencing of both strands to achieve a minimum of 3 × coverage per base position. The nucleotide sequences were subjected to virtual RFLP analysis using *i*PhyClassifier (Zhao *et al.*, 2009b) and to further analyses using CLUSTAL implemented in the software MEGA4 (Tamura *et al.*, 2007) and CLUSTAL version 5 from the LaserGene software MEGALIGN program (DNASTAR, Madison, WI, USA).

New phytoplasma subgroup 16SrIII-V

Results from *i*PhyClassifier computer-simulated, virtual RFLP analysis of 16S rRNA gene from strain PassWB-Br4 (accession no. GU292082) were in agreement with actual enzymic RFLP analysis of the rRNA gene and confirmed that this strain is a member of group 16SrIII (X-disease phytoplasma group) (Fig. 1). This finding is in agreement with the results of Davis *et al.* (1994) and Ribeiro (2008), who reported association of group 16SrIII phytoplasma strains with disease in passion fruit plants. PassWB-Br4 16S rRNA gene shared 98.96 % sequence identity with that of X-disease phytoplasma (accession no. L04682); the most similar collective RFLP pattern of the 16S rRNA gene in *i*PhyClassifier was the reference pattern for subgroup 16SrIII-B (accession no. AF189288), with a similarity coefficient of 0.94. These results indicated that strain PassWB-Br4 represents a previously undescribed subgroup in group 16SrIII. The new subgroup is designated 16SrIII-V. The enzyme used in virtual RFLP analyses that distinguished strain PassWB-Br4 from strains affiliated with other group 16SrIII subgroups in the *i*PhyClassifier database was *Dra*I.

Novel '*Candidatus* Phytoplasma' taxon

Results from *i*PhyClassifier analysis of virtual RFLP patterns (Fig. 2) of the 16S rRNA gene (accession no. GU292081) indicated that strain PassWB-Br3 represents a previously undescribed subgroup in group 16SrVI, exhibiting patterns different from, but most similar to (coefficient of similarity 0.93), those of the rRNA gene from subgroup 16SrVI-D (accession no. X83431, brinjal little leaf phytoplasma). Based on these data, strain PassWB-Br3 was designated a representative of a new subgroup, 16SrVI-I. Enzymes that distinguished the PassWB-Br3 F2n/R2 fragment from that of other group 16SrVI subgroups included *Hae*III and *Taq*I.

Prior to the present study, a partial 16S rRNA gene sequence was reported for only one phytoplasma infecting passion fruit, the passion fruit proliferation (PFP) phytoplasma (accession no. EU423902) (Ribeiro, 2008; Ribeiro *et al.*, 2008). Strain PFP was reported as a member of subgroup 16SrIII-B (Ribeiro, 2008). Thus, available data indicate that passion fruit in Brazil may be infected by at least three distinct phytoplasmas: strain PassWB-Br3 (member of subgroup 16SrVI-I; this study); strain PassWB-Br4 (member of subgroup 16SrIII-V; this study); and strain PFP (member of subgroup 16SrIII-B) (Ribeiro, 2008).

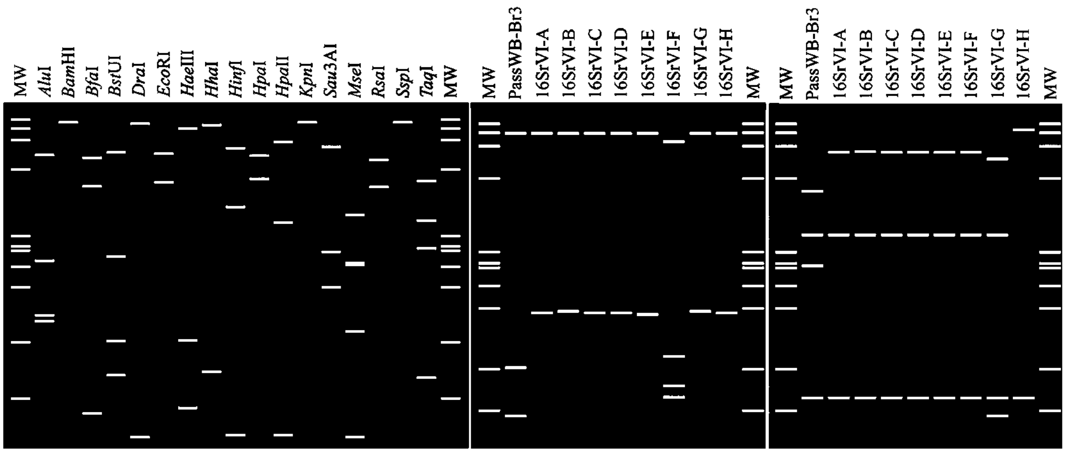

Fig. 2. Virtual RFLP patterns derived from *in silico* digestions, using *i*PhyClassifier, of F2n/R2 fragments of 16S rRNA gene from strain PassWB-Br3 (accession no. GU292081) using 17 restriction endonuclease enzymes (left): *Alu*I, *Bam*HI, *Bfa*I, *Bst*UI, *Dra*I, *Eco*RI, *Hae*III, *Hha*I, *Hin*fI, *Hpa*I, *Hpa*II, *Kpn*I, *Sau*3AI, *Mse*I, *Rsa*I, *Ssp*I, and *Taq*I. Virtual *Hae*III (centre) and *Taq*I (right) RFLP patterns distinguishing strain PassWB-Br3 from other strains in group 16SrVI. The subgroups are represented by the following strains; accession nos and strain designations are in parentheses: VI-A (AY390261, CP), VI-B (AF190224, MC, rrnA), VI-C (AF409070, EY-IL, VI-D (X83431, BLL-In-X83431), VI-E (AY270156, CSV1), VI-F (EF186819, CPS), VI-G (AF190225, MC, rrnB), and VI-H (EF651786, PLL-Ind). The restriction fragments were resolved by *in silico* electrophoresis through 3 % agarose gel. MW, ΦX174 DNA–*Hae*III digest.

To confirm whether strain PassWB-Br3 may represent a distinct species, we used the MEGALIGN option of the program DNASTAR and *i*PhyClassifier to align 16S rRNA gene sequences and calculate sequence similarity (%; identity). A 1375-base fragment (positions 94–1468; accession no. GU292081) of the 16S rRNA gene of strain PassWB-Br3 shared less than 97.5 % sequence identity with corresponding fragments of the 16S rRNA gene from all previously described '*Ca.* Phytoplasma' species. Therefore, in accordance with guidelines for the recognition of '*Ca.* Phytoplasma' species (IRPCM Phytoplasma/Spiroplasma Working Team – Phytoplasma Taxonomy Group, 2004), we propose that strain PassWB-Br3 be designated a representative of a novel, distinct '*Candidatus*' taxon, '*Candidatus* Phytoplasma sudamericanum'.

A phylogenetic tree was constructed based on 16S rRNA gene sequences from all previously described '*Ca.* Phytoplasma species', the proposed '*Ca.* Phytoplasma sudamericanum', and *Acholeplasma palmae* (Table 1). Phylogenetic analysis indicated that '*Ca.* Phytoplasma sudamericanum', '*Ca.* Phytoplasma fraxini', and '*Ca.* Phytoplasma trifolii' shared a common ancestor (Fig. 3). '*Ca.* Phytoplasma sudamericanum' formed a well-supported branch representing a distinct lineage.

Table 1. Phytoplasma strains and accession numbers of rDNAs used in this study

References noting the listed phytoplasmas include Al-Saady *et al.* (2008), Arocha *et al.* (2005, 2007), IRPCM Phytoplasma/Spiroplasma Working Team – Phytoplasma Taxonomy Group, 2004, Lee *et al.* (2006, 2011), Malembic-Maher *et al.* (2011), Schneider *et al.* (2005), Valiunas *et al.* (2006), Zhao *et al.* (2009a) and this study.

Phytoplasma	GenBank accession no.	16Sr group and subgroup*
'*Ca.* Phytoplasma sudamericanum'	GU292081	16SrVI-I
Passion fruit strain PassWB-Br4	GU292082	16SrIII-V
'*Ca.* Phytoplasma asteris'	M30790	16SrI-B
'*Ca.* Phytoplasma aurantifolia'	U15442	16SrII-B
'*Ca.* Phytoplasma australasia'	Y10097	16SrII-D
'*Ca.* Phytoplasma ulmi'	AY197655	16SrV-A
'*Ca.* Phytoplasma ziziphi'	AB052876	16SrV-B
'*Ca.* Phytoplasma rubi'	AY197648	16SrV-E
'*Ca.* Phytoplasma trifolii'	AY390261	16SrVI-A
'*Ca.* Phytoplasma fraxini'	AF092209	16SrVII-A
'*Ca.* Phytoplasma phoenicium'	AF515636	16SrIX-D
'*Ca.* Phytoplasma mali'	AJ542541	16SrX-A
'*Ca.* Phytoplasma pyri'	AJ542543	16SrX-C
'*Ca.* Phytoplasma spartii'	X92869	16SrX-D
'*Ca.* Phytoplasma prunorum'	AJ542544	16SrX-F
'*Ca.* Phytoplasma oryzae'	AB052873	16SrXI-A
'*Ca.* Phytoplasma australiense'	L76865	16SrXII-B
'*Ca.* Phytoplasma japonicum'	AB010425	16SrXII-D
'*Ca.* Phytoplasma fragariae'	DQ086423	16SrXII-E
'*Ca.* Phytoplasma cynodontis'	AJ550984	16SrXIV-A
'*Ca.* Phytoplasma brasiliense'	AF147708	16SrXV-A
'*Ca.* Phytoplasma graminis'	AY725228	16SrXVI-A
'*Ca.* Phytoplasma caricae'	AY725234	16SrXVII-A
'*Ca.* Phytoplasma americanum'	DQ174122	16SrXVIII-A
'*Ca.* Phytoplasma castaneae'	AB054986	16SrXIX-A
'*Ca.* Phytoplasma rhamni'	X76431	16SrXX-A
'*Ca.* Phytoplasma pini'	AJ632155	16SrXXI-A
'*Ca.* Phytoplasma allocasuarinae'	AY135523	ND
'*Ca.* Phytoplasma lycopersici'	EF199549	ND
'*Ca.* Phytoplasma omanense'	EF666051	16SrXXIX-A
'*Ca.* Phytoplasma tamaricis'	FJ432664	16SrXXX
'*Ca.* Phytoplasma costaricanum'	HQ225630	16SrXXXI-A

*16S rRNA gene RFLP group and subgroup classification of phytoplasmas according to Wei *et al.* (2007), Zhao *et al.* (2009a) and Lee *et al.* (2011). Group is indicated by a Roman numeral; subgroup is indicated by a letter. ND, Not determined.

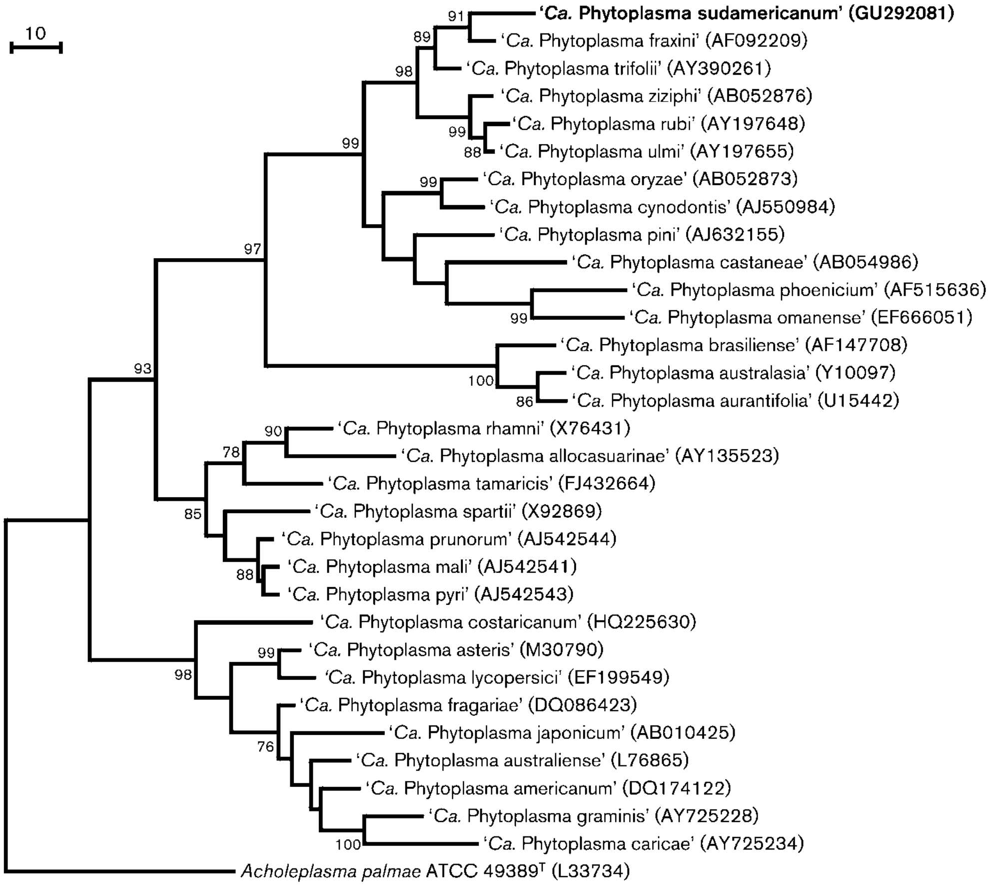

Fig. 3. Phylogenetic tree inferred from analysis of 16S rRNA gene sequences. Maximum-parsimony analysis was conducted using the close neighbour interchange (CNI) algorithm implemented in the software package MEGA4. The initial tree for the CNI search was obtained with the random addition of sequences (10 replicates). The reliability of the analysis was subjected to a bootstrap test with 1000 replicates. The percentages of replicate trees in which the associated taxa clustered together in the bootstrap test are shown next to the branches. The taxa used in the phylogenetic tree construction included reference strains of 30 previously described '*Ca.* Phytoplasma' species and '*Ca.* Phytoplasma sudamericanum' proposed in this communication (in bold). *Acholeplasma palmae* served as an outgroup during the phylogenetic tree construction. Bar, 10 nt substitutions.

Description of '*Candidatus* Phytoplasma sudamericanum'

'*Candidatus* Phytoplasma sudamericanum' [sud.a.me.ri′ca.num. French n. *sud* south; N.L. neut. adj. *americanum* American; N.L. neut. adj. *sudamericanum* pertaining to 'Amérique du Sud' (South America)].

PassWB-Br3^{R} is the reference strain.

[(Mollicutes) NC; NA; O, wall-less; NAS (GenBank accession number GU292081), oligonucleotide sequences of unique regions in the 16S rRNA gene 5′-CGAGGACAACAACTG-3′ (127–141), 5′-AGGTAAGTCTATAATTTAATTTAATTTCAGTGCTTAACGCTGTCGTGTT-3′ (580–623), 5′-AGAGACACAGGT-3′ (1018–1029) and 5′-TTGTCGTTAATTGCCAGCACAT-3′ (1095–1116); P (*Passiflora edulis* f. *flavicarpa* Deg., phloem); M]. Davis *et al.*, this study.

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