'*Candidatus* Phytoplasma costaricanum' a novel phytoplasma associated with an emerging disease in soybean (*Glycine max*)

I.-M. Lee,[1] K. D. Bottner-Parker,[1] Y. Zhao,[1] W. Villalobos[2] and L. Moreira[2,3]

Correspondence
I.-M. Lee
ingming.lee@ars.usda.gov

[1]Molecular Plant Pathology Laboratory, USDA-ARS, Beltsville, MD 20705, USA

[2]Centro de Investigación en Biologia Celular y Molecular, Universidad de Costa Rica (UCR), CP 11501-2060, San José, Costa Rica

[3]Escuela de Agronomía, UCR, San José, Costa Rica

A novel phytoplasma, designated strain SoyST1c1, associated with a newly emerging disease in soybean (*Glycine max*), known as soybean stunt (SoyST), was found in 2002 in a soybean plantation in Alajuela Province, Costa Rica. The same phytoplasma, or a very closely related strain, also infected sweet pepper (*Capsicum annuum*) with purple vein syndrome (SwPPV) and passion fruit vine (*Passiflora edulis*) with bud proliferation disease (PasFBP) in the same region. Sequence analysis of cloned 16S rRNA gene sequences (GenBank accession nos FJ226068–FJ226073 and HQ225624–HQ225635) indicated that all three affected plants were infected by phytoplasmas that shared <97.5 % sequence similarity with previously described phytoplasmas. The SoyST-causing phytoplasma represents a new taxon, most closely related to phytoplasma group 16SrI and 16SrXII strains. Virtual RFLP analysis indicated that the SoyST-causing phytoplasma and its closely related strains represent a novel 16Sr group, designated 16SrXXXI. Phylogenetic analysis of 16S rRNA gene sequences from the new phytoplasma strains, those previously described as '*Candidatus* Phytoplasma spp.' and other distinct, as yet unnamed, phytoplasmas indicated that the SoyST-causing phytoplasma represents a distinct lineage within the aster yellows/stolbur branch on the phylogenetic tree. On the basis of its unique 16S rRNA gene sequence and biological properties, strain SoyST1c1 represents a novel taxon, for which the name '*Candidatus* Phytoplasma costaricanum' is proposed with SoyST1c1 as the reference strain.

Phytoplasmas are unculturable plant-pathogenic, wall-less bacteria that cause diseases in several hundred species of plant worldwide (Bertaccini, 2007; McCoy *et al.*, 1989; Lee *et al.*, 2000; Hogenhout *et al.*, 2008). Phytoplasmas constitute a large, genetically diverse group. Currently, 30 groups and more than 50 subgroups have been classified based on RFLP analyses of 16S rRNA gene sequences and 28 taxa in the genus '*Candidatus* Phytoplasma' have been reported (IRPCM, 2004; Lee *et al.*, 2000; Wei *et al.*, 2007, 2008; Zhao *et al.*, 2009a, b). In 2002, an outbreak of a new disease affecting soybean (*Glycine max*) occurred in a soybean plantation in Alajuela Province, Costa Rica (Villalobos *et al.*, 2009). The infected soybean plants exhibited symptoms that included general stunting, small leaves, excessive bud breaking and aborted seed pods, which are characteristic of aster yellows symptoms caused by phytoplasma infection. In addition, infected soybean plants remained green at harvest time. A new phytoplasma was detected in all samples of symptomatic plants tested. Phytoplasmas closely related to the soybean phytoplasma were also found to be associated with sweet pepper (*Capsicum annuum*) and passion fruit vines (*Passiflora edulis*) in the same region. The infected sweet pepper plants exhibited purple vein syndrome (SwPPV), characterized by dark green and rugose leaves, a zigzag pattern to the midvein and purple vein discoloration; the infected passion fruit vine exhibited bud proliferation (PasFBP) and chlorosis (Villalobos *et al.*, 2009). In this study, we propose that the novel phytoplasma be elected as a novel taxon in the genus '*Candidatus* Phytoplasma', with the name '*Candidatus* Phytoplasma costaricanum'.

Abbreviations: PasFBP, passion fruit vine bud proliferation disease; SoyST, soybean stunt; SwPPV, sweet pepper purple vein syndrome.

The GenBank/EMBL/DDBJ accession number for the 16S rRNA gene sequence of the reference strain, SoyST1c1, is HQ225630. GenBank/EMBL/DDBJ accession numbers for other sequences determined in this study are given in Fig. 1.

Total nucleic acid was extracted from leaf vein tissue or buds according to previously described methods (Lee *et al.*, 1998). Putative phytoplasmas associated with diseased soybean (four plants), sweet pepper (three plants) and

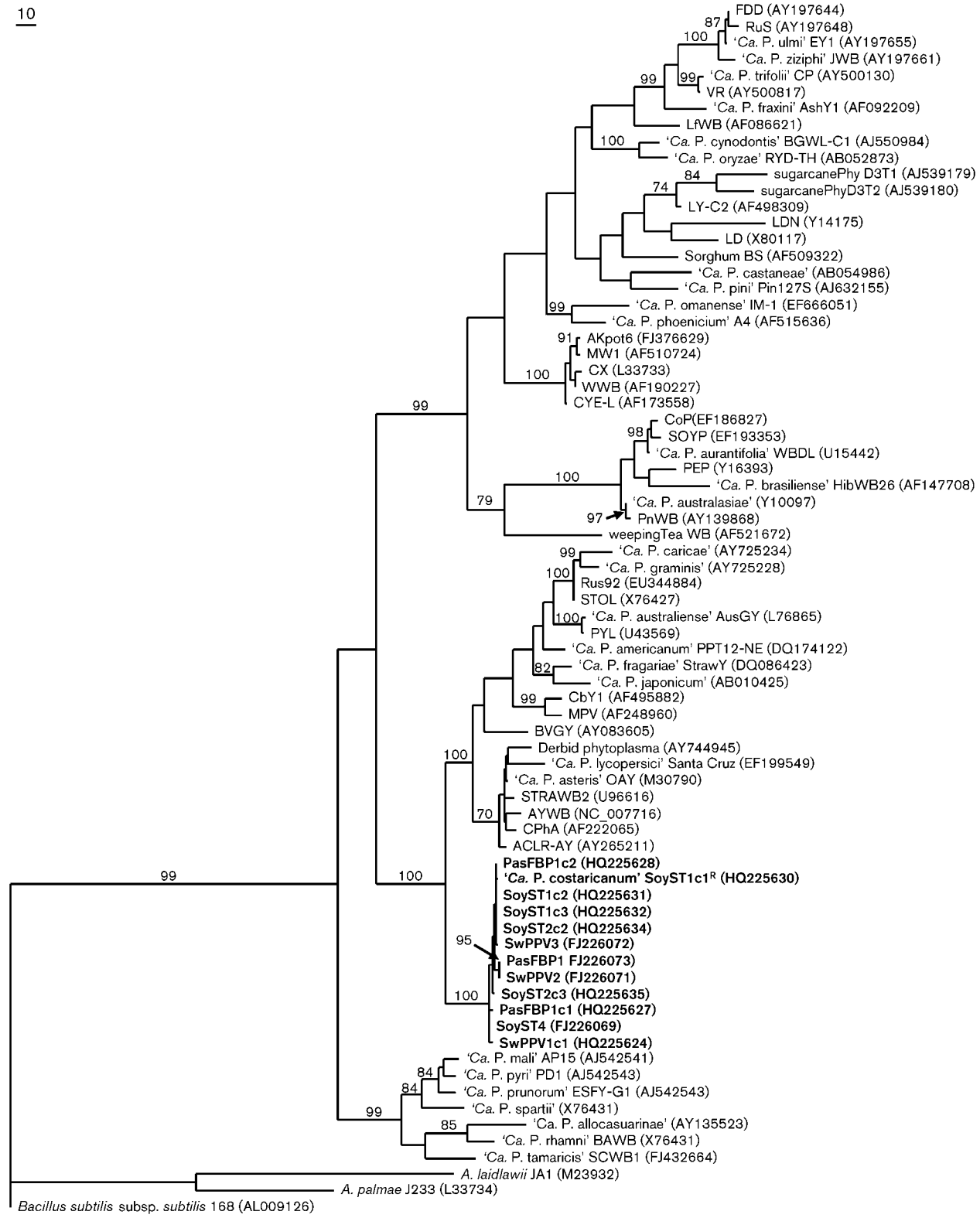

Fig. 1. Phylogenetic tree constructed by parsimony analysis of nearly full-length 16S rRNA gene sequences from previously described taxa of the genus '*Candidatus* Phytoplasma', representative phytoplasma strains from each proposed 16Sr group, representative strains of the novel putative '*Candidatus* Phytoplasma costaricanum' taxon (indicated in bold) and *Acholeplasma* spp. Sequences were aligned using CLUSTAL W. *Bacillus subtilis* subsp. *subtilis* strain 168 (AL009126) was used as the outgroup to root the tree. Branch lengths are proportional to the number of inferred character state transformations. Bootstrap values >70 %

Fig. 1 cont. (measures of support for the inferred subclades) are shown on branches. GenBank accession numbers are given in parentheses. Bar, 10 substitutions per nucleotide position. Phytoplasma strain abbreviations: FDD (16SrV-C), Flavescence dorée; RuS (16SrV-E), *Rubus* stunt; VR (16SrV-A), vinca virescence; LfWB (16SrVII-A), loofah witches'-broom; LY-C2 (16SrIV-A), coconut lethal yellowing; LDN (16SrXXII-A), coconut lethal yellowing Nigerian Awka disease; LD, coconut lethal yellowing Tanzanian lethal disease; sorghum BS (16SrXXIV-A), sorghum bunchy shoot; AKpot6 (16SrIII-N), Alaskan potato purple top; MW1 (16SrIII-F), milkweed yellows; CYE-L (16SrIII-B), clover yellow edge; CX (16SrIII-A), Canadian peach X; WWB (16SrIII-G), walnut witches'-broom; CoP (16SrII-C), cotton phyllody; SoyP (16SrII-C), soybean phyllody; PnWB (16SrII-A), peanut witches'-broom; PEP (16SrII-E), *Picris echioides* phyllody; weeping tea WB (16SrXXV-A), weeping tea tree witches'-broom; Rus92 (16SrXII), Russian potato purple top; PYL (16SrXII), *Phormium* yellow leaf; STOL (16SrXII-A), sweet pepper stolbur in periwinkle; CbY1 (16SrXIII), China berry yellows; MPV (XIII-A), Mexican periwinkle virescence; BVGY (16SrXII-A), Buckland valley grapevine yellows; AYWB (16SrI-A), aster yellows witches'-broom; CPhA (16SrI-C), clover phyllody; STRAWB2 (16SrI-K), strawberry 2 phytoplasma; ACLR-AY (16SrI-F), apricot chlorotic leaf roll; PasFBP (16SrXXXI), passion fruit bud proliferation; SoyST (16SrXXXI), soybean stunt; SwPPV (16SrXXXI), sweet pepper purple vein.

passion fruit vine (one plant) were detected by using nested PCR assays with the phytoplasma-specific universal primer pair P1/16S-SR followed by primer pair R16F2n/R16R2, according to previously described procedures (Gundersen & Lee, 1996; Lee *et al.*, 2004). RFLP analyses using the restriction enzymes *Alu*I, *Mse*I, *Hha*I, *Rsa*I, *Hpa*II and *BstU*I (*Tha*I) followed by separation through a 5 % polyacrylamide gel was performed and the collective RFLP patterns indicated that all samples from symptomatic plants were associated with a novel phytoplasma.

Nearly full-length 16S rRNA gene sequences of phytoplasmas (P1/16S-SR PCR amplicons) obtained from infected soybean, sweet pepper and passion fruit vines were purified using Quantum Prep PCR Kleen Spin Columns (Bio-Rad) and cloned into *Escherichia coli* using the TOPO-TA cloning kit (Invitrogen) according to the manufacturers' instructions. Sequencing was performed with an automated DNA sequencer (ABI Prism model 3100) at the Center for Biosystems Research (University of Maryland, College Park, MD, USA).

Sequences were aligned using CLUSTAL W (Higgins & Sharp, 1989) and sequence similarities were calculated using the MEGALIGN program from the LASERGENE software suite. Sequences were deposited in GenBank, the accession numbers of which are given in Fig. 1. Other sequences used in this study were obtained from GenBank. 16S rRNA gene sequence comparisons revealed that the novel phytoplasma, termed soybean stunt (SoyST) phytoplasma, represented a novel taxon, sharing <97.5 % sequence similarity with previously described phytoplasmas, including the 28 taxa of the genus '*Candidatus* Phytoplasma' already named.

Phylogenetic analysis based on 16S rRNA gene sequences, including those of the SoyST phytoplasma, the closely related strains associated with infected sweet pepper and passion fruit vine, all the '*Candidatus* Phytoplasma' taxa reported to date, representatives of each 16Sr phytoplasma group, *Acholeplasma* spp., and other low G+C Gram-positive bacteria was performed to determine the phylogenetic position of this novel phytoplasma.

Cladistic analyses were performed with PAUP version 4.0 (Swofford, 2002). Uninformative characters were excluded from the analyses. A phylogenetic tree was reconstructed using a heuristic search (or neighbour-joining algorithm) via random stepwise addition, implementing the tree bisection and reconnection branch-swapping algorithm to find the optimal tree(s) (Lee *et al.*, 2006; Martini *et al.*, 2007). *Bacillus subtilis* was selected as the out-group to root the tree. The analysis was replicated 1000 times. Bootstrapping was performed to estimate stability and support for the inferred clades. The analysis resulted in 300 equally parsimonious trees. Based on the consensus tree, a representative parsimonious tree was inferred (Fig. 1). The phytoplasma clade formed three major phylogenetic groups. Strains of the new SoyST phytoplasma and the closely related strains SwPPV and PasFBP formed a discreet monophyletic group within

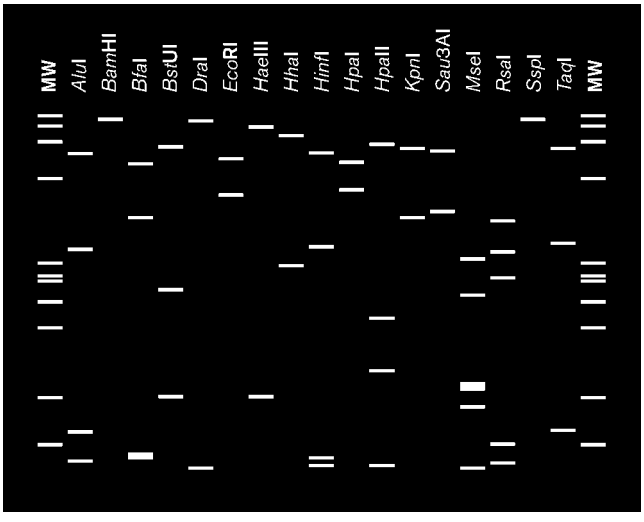

Fig. 2. Collective virtual RFLP patterns derived from *in silico* digestions of 16S rRNA gene R16F2n/R16R2 fragments from the new 16SrXXXI group of phytoplasmas. Recognition sites for 17 restriction enzymes were used in the simulated digestions. MW, molecular weight marker (derived from øX174 RFI DNA *Hae*III digest); fragment sizes (bp) from top to bottom: 1353, 1078, 872, 603, 310, 281, 271, 234, 194, 118, 72.

the major phylogenetic group, which comprised strains belonging to eight 16Sr groups: 16SrI, 16SrXII, 16SrXIII, 16SrXVI, 16SrXVII, 16SrXVIII, 16SrXXIII and 16SrXXVIII. A new group, 16SrXXXI was established to represent the SoyST phytoplasmas and their closely related strains. The closest relative of the novel phytoplasmas was '*Candidatus* Phytoplasma asteris' (subgroup 16SrI-C), sharing 96.4 % 16S rRNA gene sequence similarity with the novel phytoplasma.

Collective RFLP patterns using 17 restriction enzymes were generated for members of the new phytoplasma group 16SrXXXI by using virtual gel analysis in the *i*Phyclassifier program (Zhao *et al.*, 2009b) (Fig. 2). The new 16SrXXXI group could be differentiated from 30 other phytoplasma groups previously characterized by analysis using several restriction enzymes (data not shown). Group 16SrXXXI could be differentiated from the most closely related groups, 16SrI and 16SrXII, by analyses using restriction enzymes *Alu*I and *Mse*I (Fig. 3). The virtual RFLP patterns were consistent with those shown on actual gel analysis (Fig. 4). Sequence variations (0.1–0.8 %) were detected between the reference strain of '*Candidatus* Phytoplasma costaricanum' (SoyST1c1) and related strains associated with SoyST, SwPPV, and PasFBP diseases as shown in Fig. 4. Further study is needed to determine whether some strains represent variants of the reference strain or different subgroups within group 16SrXXXI based on virtual gel analysis.

According to the recommendations of the International Research Program for Comparative Mycoplasmology (IRPCM) Phytoplasma/Spiroplasma Working Team – Phytoplasma Taxonomy Group (IRPCM, 2004), ''a '*Ca.* Phytoplasma' species description should refer to a single, unique 16S rRNA gene sequence (>1200 bp)'' and ''a strain can be recognized as a novel '*Ca.* Phytoplasma' species if its 16S rRNA gene sequence has <97.5 % similarity to that of any previously described '*Ca.* Phytoplasma' species''. Results from the sequence and phylogenetic analyses conducted in this study support the recognition of the novel SoyST phytoplasma as a new taxon of the genus '*Candidatus* Phytoplasma'. On the basis of unique DNA and biological properties, we propose that the SoyST phytoplasmas represents a new taxon, '*Candidatus* Phytoplasma costaricanum', with SoyST1c1 as the reference strain.

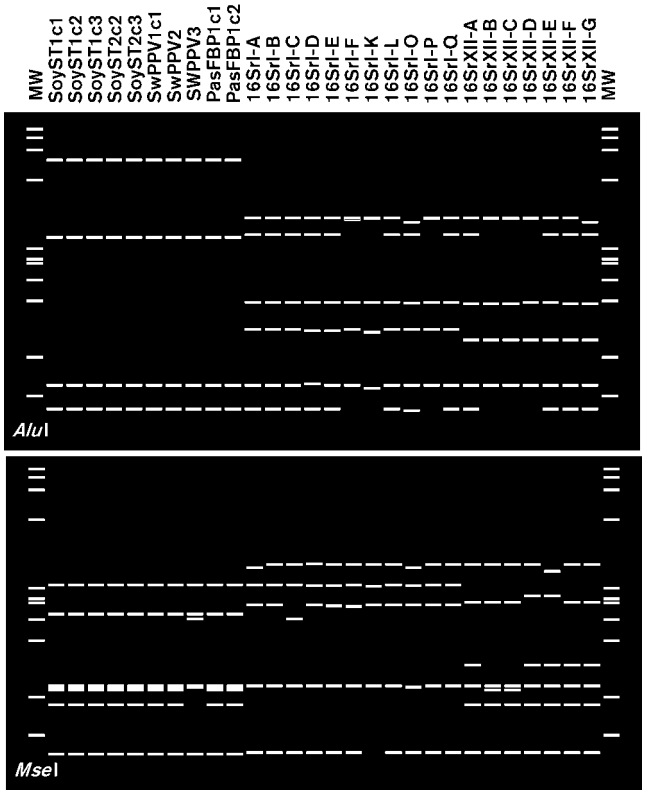

Fig. 3. Computer-simulated virtual RFLP patterns derived from *in silico* digestions of phytoplasma 16S rRNA gene R16F2n/R16R2 fragments (~1.2 kb in length) from the new 16SrXXXI group of phytoplasmas and representative strains of groups 16SrI and 16SrXII with two key enzymes: *Alu*I (top) and *Mse*I (bottom). MW, molecular weight marker (derived from øX174 RFI DNA *Hae*III digest); fragment sizes (bp) from top to bottom: 1353, 1078, 872, 603, 310, 281, 271, 234, 194, 118, 72.

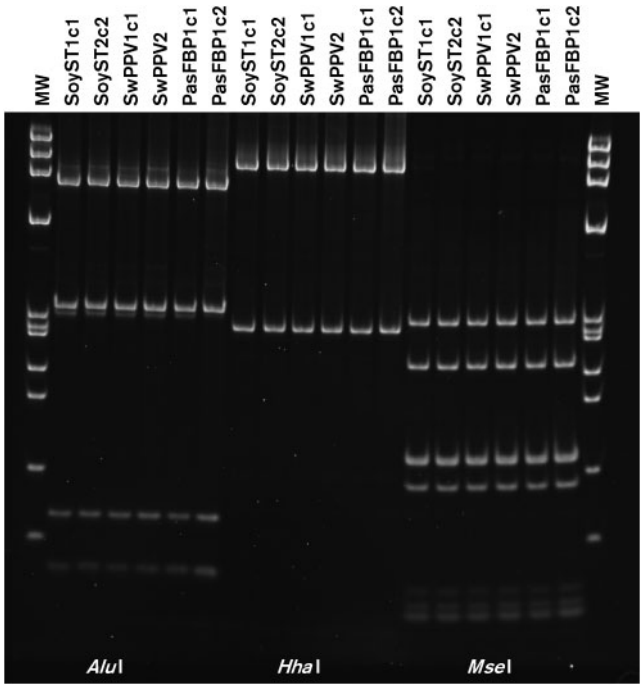

Fig. 4. RFLP patterns derived from actual gel analysis of phytoplasma 16S rRNA gene R16F2n/R16R2 fragments (~1.2 kb) from the new 16SrXXXI group of phytoplasmas digested using restriction enzymes *Alu*I, *Hha*I and *Mse*I and separated by electrophoresis through a 5 % polyacrylamide gel. MW, molecular weight marker (derived from øX174 RFI DNA *Hae*III digest); fragment sizes (bp) from top to bottom: 1353, 1078, 872, 603, 310, 281, 271, 234, 194, 118, 72.

Description of '*Candidatus* Phytoplasma costaricanum'

'*Candidatus* Phytoplasma costaricanum' (cos.ta.ri.ca′num. N.L. neut. adj. *costaricanum* pertaining to Costa Rica).

SoyST1c1 is the reference strain (GenBank accession number HQ225630). Related phytoplasma strains include: SoyST1, SoyST4, SoyST1c2, SoyST1c3, SoyST2c1, SoyST2c2 and SoyST2c3, associated with soybean stunt disease; SwPPV1, SwPPV2, SwPPV3, SwPPV1c1, SwPPV1c2 and SwPPV1c3, associated with sweet pepper purple vein disease; and PasFBP1, PasFBP1c1, PasFBP1c2 and PasFBP1c3, associated with passion fruit vine bud proliferation disease, in Costa Rica. The vectors that transmit the novel phytoplasma are unknown.

'*Candidatus* Phytoplasma costaricanum' [(Mollicutes) NC; NA; O, wall-less; NAS (GenBank accession numbers FJ226068-FJ226073 and HQ225624-HQ225635), oligonucleotide sequences of unique regions of the 16S rRNA gene: TTAAGGAAGAAAAATTGGTGGAAA, TTAGGTAAGTTTATGGTGTAA, GTTCAACGCTTAACGTTGTGATG, CTACAACGCAAGTTGATG GGGGGCCTAACTCGCAAGA. P (*Glycine max*, phloem); M].

References

Bertaccini, A. (2007). Phytoplasmas: diversity, taxonomy, and epidemiology. *Front Biosci* **12**, 673–689.

Gundersen, D. E. & Lee, I.-M. (1996). Ultrasensitive detection of phytoplasmas by nested-PCR assays using two universal primer pairs. *Phytopathol Mediterr* **35**, 144–154.

Higgins, D. G. & Sharp, P. M. (1989). Fast and sensitive multiple sequence alignments on a microcomputer. *Comput Appl Biosci* **5**, 151–153.

Hogenhout, S. A., Oshima, K., Ammar, D., Kakizawa, S., Kingdom, H. N. & Namba, S. (2008). Phytoplasmas: bacteria that manipulate plants and insects. *Mol Plant Pathol* **9**, 403–423.

IRPCM & Phytoplasma/Spiroplasma Working Team – Phytoplasma Taxonomy Group (2004). '*Candidatus* Phytoplasma', a taxon for the wall-less, non-helical prokaryotes that colonize plant phloem and insects. *Int J Syst Evol Microbiol* **54**, 1243–1255.

Lee, I.-M., Gundersen-Rindal, D. E., Davis, R. E. & Bartoszyk, I. M. (1998). Revised classification scheme of phytoplasmas based on RFLP analyses of 16S rRNA and ribosomal protein gene sequences. *Int J Syst Bacteriol* **48**, 1153–1169.

Lee, I.-M., Davis, R. E. & Gundersen-Rindal, D. E. (2000). Phytoplasma: phytopathogenic mollicutes. *Annu Rev Microbiol* **54**, 221–255.

Lee, I.-M., Martini, M., Marcone, C. & Zhu, S. F. (2004). Classification of phytoplasma strains in the elm yellows group (16SrV) and proposal of '*Candidatus* Phytoplasma ulmi' for the phytoplasma associated with elm yellows. *Int J Syst Evol Microbiol* **54**, 337–347.

Lee, I.-M., Bottner, K. D., Secor, G. & Rivera-Varas, V. (2006). '*Candidatus* Phytoplasma americanum', a phytoplasma associated with a potato purple top wilt disease complex. *Int J Syst Evol Microbiol* **56**, 1593–1597.

Martini, M., Lee, I.-M., Bottner, K. D., Zhao, Y., Botti, S., Bertaccini, A., Harrison, N. A., Carraro, L., Marcone, C. & other authors (2007). Ribosomal protein gene-based phylogeny for finer differentiation and classification of phytoplasmas. *Int J Syst Evol Microbiol* **57**, 2037–2051.

McCoy, R. E., Caudwell, A., Chang, C. J., Chen, T.-A., Chiykowski, L. N., Cousin, M. T., Dale, J. L., DeLeeuw, G. T. N., Golino, D. A. & other authors (1989). Plant diseases associated with mycoplasma-like organisms. In *The Mycoplasmas*, vol. V, pp. 545–640. Edited by R. F. Whitcomb & J. G. Tully. New York: Academic Press.

Swofford, D. L. (2002). PAUP*: Phylogenetic analysis using parsimony (and other methods), version 4. Sunderland, MA: Sinauer Associates.

Villalobos, W., Moreira, L., Rivera, C. & Lee, I.-M. (2009). First report of new phytoplasma diseases associated with soybean, sweet pepper, and passion fruit in Costa Rica. *Plant Dis* **93**, 201.

Wei, W., Davis, R. E., Lee, I.-M. & Zhao, Y. (2007). Computer-simulated RFLP analysis of 16S rRNA genes: identification of ten new phytoplasma groups. *Int J Syst Evol Microbiol* **57**, 1855–1867.

Wei, W., Lee, I.-M., Davis, R. E., Suo, X. & Zhao, Y. (2008). Automated RFLP pattern comparison and similarity coefficient calculation for rapid delineation of new and distinct phytoplasma 16Sr subgroup lineages. *Int J Syst Evol Microbiol* **58**, 2368–2377.

Zhao, Y., Sun, Q., Wei, W., Davis, R. E., Wu, W. & Liu, Q. (2009a). '*Candidatus* Phytoplasma tamaricis', a novel taxon discovered in witches'-broom-diseased salt cedar (*Tamarix chinensis* Lour.). *Int J Syst Evol Microbiol* **59**, 2496–2504.

Zhao, Y., Wei, W., Lee, I.-M., Shao, J., Suo, X. & Davis, R. E. (2009b). Construction of an interactive online phytoplasma classification tool, *i*PhyClassifier, and its application in analysis of the peach X-disease phytoplasma group (16SrIII). *Int J Syst Evol Microbiol* **59**, 2582–2593.