# 'Candidatus Phytoplasma graminis' and 'Candidatus Phytoplasma caricae', two novel phytoplasmas associated with diseases of sugarcane, weeds and papaya in Cuba

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During 2003, surveys of sugarcane yellow leaf disease and papaya bunchy top-like disease were carried out on plantations in Havana province, Cuba, to determine the roles of weeds and *Auchenorrhyncha* insects in the epidemiology of these diseases. More than 250 plant and insect samples were collected and indexed by using a nested PCR for phytoplasma 16S rDNA with the generic primer pairs P1/P7 and R16F2n/R16R2. The PCR products were further characterized by restriction fragment length polymorphism using *Hae*III, *Alu*I, *Sau*3AI, *Tru*9I, *Hha*I, *Hpa*II and *Taq*I endonucleases, giving patterns that distinguished them from those of the other reference phytoplasmas analysed. Phylogenetic analysis of 16S rRNA gene sequences identified the phytoplasmas present in sugarcane (*Saccharum officinarum* L.), *Cynodon dactylon* L., *Conyza canadensis* L. Cronq., S*orghum halepense* L. Pers., *Macroptilium lathyroides* L. Urb., *Saccharosydne saccharivora* (Westwood) and *Cedusa* spp., and those in papaya (*Carica papaya* L.) and *Empoasca papayae*, as two novel provisional phytoplasma species. We propose that these phytoplasmas should be given *Candidatus* status, as '*Candidatus* Phytoplasma graminis' and '*Candidatus* Phytoplasma caricae', respectively.

## INTRODUCTION

Sugarcane (*Saccharum officinarum* L.) is the most economically important crop in Cuba, where the control of pests and diseases is constrained by low-input farming systems, whereas papaya (*Carica papaya* L.) has been identified for development as an export crop with an estimated production of 55 000–60 000 tonnes per year. Yellow leaf syndrome (YLS) is one of five main diseases affecting Cuban sugarcane production (Peralta *et al.*, 1999) and is associated with phytoplasmas (Arocha *et al.*, 1999). The delphacid planthopper *Saccharosydne saccharivora* was recently identified as a vector of YLS, and phytoplasmas were shown to be the causal agent of the disease in Cuba (Arocha *et al.*, 2005b). However, since phytoplasmas are naturally transmitted by many *Auchenorryncha* leafhoppers and planthoppers (Carraro *et al.*, 2001; Gatineau *et al.*, 2001; Lee *et al.*, 2003), other species could play a role in the epidemiology of the disease.

Papaya bunchy top (PBT) disease has been associated with a bacterium-like organism from the α-1 subgroup of the *Proteobacteria* in the genus *Rickettsia*, and is naturally spread by the leafhopper *Empoasca papayae* Oman (Davis *et al.*, 1998). Mosaic, yellow crinkle and dieback diseases of papaya in Australia are known to be associated with phytoplasmas (Gibb *et al.*, 1996, 1998; White *et al.*, 1998; De La Rue *et al.*, 1999), and are the main phytosanitary problems of the Australian papaya industry, causing losses of 100 % in some

Abbreviations: nPCR, nested PCR; PBT, papaya bunchy top; RFLP, restriction fragment length polymorphism; YLS, yellow leaf syndrome.

The GenBank/EMBL/DDBJ accession numbers for the 16S rRNA gene sequences reported in this paper are given in Table 1.

plantations (Guthrie *et al.*, 1998). Epidemiological studies in Australia have identified *Orosius* leafhoppers species as target candidates for transmission studies (Padovan & Gibb, 2001). In Cuba, recent reports from papaya-growing areas have confirmed that PBT is spreading (Arocha *et al.*, 2003), but no putative insect vectors have been identified as yet.

Little is known about the epidemiological role that weeds play in diseases of either sugarcane or papaya caused by phytoplasmas. In this paper, we report on the identification of phytoplasmas associated with YLS, PBT-like disease, weeds and putative vectors discovered during epidemiological studies of sugarcane and papaya plantations in Cuba.

## METHODS

**Plant, insect and reference phytoplasma strain materials.** Leaf samples from 120 sugarcane plants with and without YLS symptoms, 106 papaya plants with and without PBT-like symptoms and 50 assorted weed species with and without symptoms of white leaf and witches'-broom were collected from adjacent sugarcane and papaya plantations in Güines and Nueva Paz, Havana province, Cuba. Weeds were identified as Bermuda grass (*Cynodon dactylon* L.), Canadian horseweed (*Conyza canadensis* L. Cronq.; Asteraceae), Johnson grass (*Sorghum halepense* L. Pers.), phasey bean (*Macroptilium lathyroides* L. Urb.; Fabaceae), llanos macro (*Macroptilium longepedunculatum* Benth. Urb.; Fabaceae), coatbuttons (*Tridax procumbens* L.; Asteraceae) and itchgrass [*Rottboellia cochinchinensis* (Lour.) W. D. Clayton; Poaceae]. Twenty-one adult *Saccharosydne saccharivora* (Westwood) delphacid planthoppers and 19 planthoppers of unknown species belonging to the genus *Cedusa* were collected from YLS-affected sugarcane plantations, and 23 as-yet-unidentified species of *E. papayae* leafhoppers were trapped in neighbouring papaya fields. DNA of reference phytoplasma strains Stolbur (STOL; 16SrXII), Bois Noir (VK; 16SrXII), provided by Dr Giuseppe Firrao, and American aster yellows (AAY; 16SrI), Apricot chlorotic leaf roll, Spain (ACLR; 16SrI) and Plum leptonecrosis (PLN; 16SrX), from the phytoplasma reference collection at Rothamsted Research, UK, were used for direct comparison of restriction fragment length polymorphism (RFLP) patterns.

**DNA extraction.** DNA was extracted from 1·5 g leaf tissue and batches of three insects by using the method of Doyle & Doyle (1990). Ethanol-precipitated nucleic acids were dried, resuspended in 100 µl TE buffer (50 mM Tris/HCl, pH 8·0 and 10 mM EDTA) and incubated with RNase for 1 h at 37 °C. Aliquots of final DNA preparations were used as templates for PCR.

**DNA amplification and RFLP analysis.** A nested PCR (nPCR) assay was performed using puRe*Taq* Ready-To-Go PCR beads (Amersham Biosciences) with phytoplasma 16S rDNA primers P1 (Deng & Hiruki, 1991) and P7 (Schneider *et al.*, 1995).

PCR was done using a programmable thermocycler (MJ Research) with 30 cycles of denaturation at 95 °C for 30 s (2 min for first cycle), annealing at 53 °C for 1 min 15 s and extension at 72 °C for 1 min 30 s (10 min for the final cycle). The PCR products obtained were reamplified with 35 cycles of denaturation at 94 °C for 30 s (95 °C, 2 min for one cycle), annealing at 56 °C for 1 min and extension at 72 °C for 2 min, and a final extension step of 72 °C for 10 min, using the nested 16S rDNA primer pair R16F2n/R16R2 (Gundersen & Lee, 1996).

DNA from papaya and *E. papayae* samples were also analysed by using a PCR assay with the primer pair PBTF1/PBTR1, which amplify the common rickettsial flavoprotein subunit of the succinate dehydrogenase gene (*sdh*A). The PCR conditions used were according to Davis *et al.* (1998).

nPCR products were digested with *Hae*III, *Alu*I, *Sau*3AI, *Tru*9I, *Hpa*II, *Hha*I or *Taq*I restriction enzymes, according to the manufacturer's instructions. Digestion products were electrophoresed in 1·5 % agarose gels, and visualized after staining with ethidium bromide by UV transillumination. RFLP patterns were compared with previously published patterns (Schneider *et al.*, 1993; Lee *et al.*, 1995, 1998; Davis *et al.*, 1997; Marcone *et al.*, 1997; Montano *et al.*, 2001; Šeruga *et al.*, 2003).

**DNA sequencing.** Phytoplasma rDNA amplified by PCR using the primer pair P1/P7 was purified on spin columns (QIAquick gel extraction kit; QIAGEN). The PCR products were forward- and reverse-sequenced using primer pair P1/P7 by the Sequencing Service, School of Life Sciences, University of Dundee, UK (http://www.dnaseq.co.uk), with Applied Biosystems Big-Dye version 3.1 chemistry on an Applied Biosystems model 3730 automated capillary DNA sequencer.

**Sequence similarity, similarity coefficient calculations and putative restriction-site analysis.** The 16S rRNA gene sequences obtained were compared with others in GenBank (Table 1). Sequence editing and alignment were performed using the programs SEQED, LINEUP and PILEUP in the Wisconsin GCG version 10 package (Devereux *et al.*, 1984). Alignments of sequences were generated, sequence similarities were evaluated and putative restriction-site maps were produced with the enzymes *Hpa*II, *Taq*I, *Dra*I, *Bfa*I, *Alu*I, *Kpn*I, *Hae*III, *Tru*9I, *Sau*3AI, *Hin*fI, *Rsa*I and *Hha*I, using the RESearch program (Invitro; Rothamsted Research). Similarity coefficients ($F$) between the 16S rRNA gene sequences of phytoplasmas identified from sugarcane, weeds, papaya and putative vectors, and of other reference phytoplasmas, were calculated as described by Montano *et al.* (2001). $F$ was calculated as $F=2N_{xy}/(N_x+N_y)$, where $x$ and $y$ are the strains of two given phytoplasmas, $N_x$ and $N_y$ are the number of fragments resulting from enzymic digestion of strains $x$ and $y$, respectively, and $N_{xy}$ is the number of fragments shared by the two strains.

**Phylogenetic analysis.** Phylogenetic analysis was carried out using programs of PHYLIP version 3.5c (Felsenstein, 1993). Phylogenetic trees were constructed from the aligned sequences by using a parsimony method (DNAPARS) and 1000 bootstrap datasets generated by the program SEQBOOT from the original dataset. The consensus tree was generated by using the program CONSENSE, with *Acholeplasma laidlawii* as the outgroup sequence to root the phylogenetic tree. The consensus tree was displayed with TreeView (Page, 1996).

## RESULTS

### DNA amplification

nPCR products of about 1250 bp in size and with a typical *Hae*III phytoplasma profile (data not shown) were produced by reactions primed with DNA extracted from sugarcane, papaya, *Saccharosydne saccharivora*, *Cedusa* spp. and *E. papayae* and from all phytoplasma positive controls. Phytoplasma rDNA was also amplified from *Cynodon dactylon*, *Conyza canadensis*, *Sorghum halepense* and *Macroptilium lathyroides*. *Macroptilium lathyroides* and *Conyza canadensis* are, as far as we know, novel phytoplasma hosts. No PCR bands were observed in the negative controls. The PCR results are summarized in Table 2. PCR

**Table 1.** Acronyms, strain designation, RFLP group and GenBank accession numbers of phytoplasma 16S rRNA gene sequences used to construct the phylogenetic tree

| Acronym | Phytoplasma strain designation | RFLP group | Accession number |
|---|---|---|---|
| CP | Clover proliferation | 16SrVI | L33761 |
| '*Ca.* P. trifolii' | '*Candidatus* Phytoplasma trifolii' | 16SrVI | AY390261 |
| BLL | Brinjal little leaf | 16SrVI | X83431 |
| '*Ca.* P. fraxini' | '*Candidatus* Phytoplasma fraxini' | 16SrVII | AF092209 |
| AshY | Ash yellows | 16SrVII | X68339 |
| '*Ca.* P. ulmi' | '*Candidatus* Phytoplasma ulmi' | 16SrV | AF122910 |
| FD | Flavescence dorée | 16SrV | AF176319 |
| '*Ca.* P. ziziphi' | '*Candidatus* Phytoplasma ziziphi' | 16SrV | AY072722 |
| LWB | Loofah witches'-broom | 16SrVIII | L33764 |
| StLL | *Stylosanthes* little leaf | 16SrVIII | AJ289192 |
| BGWL | Bermuda grass white leaf | 16SrXIV | AF248961 |
| '*Ca.* P. cynodontis' | '*Candidatus* Phytoplasma cynodotis' | 16SrXIV | AJ550984 |
| SCWL | Sugarcane white leaf | 16SrXI | X76432 |
| '*Ca.* P. oryzae' | '*Candidatus* Phytoplasma oryzae' | 16SrXI | D12581 |
| FCoLY | Coconut yellows | 16SrIV | U18747 |
| CoLY | Coconut yellows | 16SrIV | AF498309 |
| YCoLD | Coconut lethal decline | 16SrIV | U18753 |
| LDT | Coconut lethal decline | 16SrIV | X80117 |
| LDN | Coconut lethal decline | 16SrIV | Y14175 |
| LDG | Coconut lethal decline | 16SrIV | Y13912 |
| '*Ca.* P. pini' | '*Candidatus* Phytoplasma pini' | 16SrIV | AJ310849 |
| '*Ca.* P. castaneae' | '*Candidatus* Phytoplasma castaneae' | 16SrIV | AB054986 |
| '*Ca.* P. phoenicium' | '*Candidatus* Phytoplasma phoenicium' | 16SrIX | AF515637 |
| PPWB | Pigeon pea witches'-broom | 16SrIX | U18763 |
| VWB | Vaccinia witches'-broom | 16SrIII | X76430 |
| WX | Western X-disease | 16SrIII | L04682 |
| PYC | Papaya yellow crinkle | 16SrII | Y10097 |
| PWB | Peanut witches'-broom | 16SrII | L33765 |
| PM | Papaya mosaic | 16SrII | Y10096 |
| '*Ca.* P. aurantifolia' | '*Candidatus* Phytoplasma aurantifolia' | 16SrII | U15442 |
| '*Ca.* P. brasiliense' | '*Candidatus* Phytoplasma brasiliense' | 16SrXV | AF147708 |
| AP | Apple proliferation | 16SrX | X68375 |
| '*Ca.* P. mali' | '*Candidatus* Phytoplasma mali' | 16SrX | AJ542541 |
| '*Ca.* P. pyri' | '*Candidatus* Phytoplasma pyri' | 16SrX | AJ542543 |
| PD | Pear decline | 16SrX | Y16392 |
| '*Ca.* P. prunorum' | '*Candidatus* Phytoplasma prunorum' | 16SrX | AJ542544 |
| '*Ca.* P. spartii' | '*Candidatus* Phytoplasma spartii' | 16SrX | X92869 |
| *Emp5* | *Empoasca* phytoplasma, sample 5 | 16SrX | AY725236 |
| '*Ca.* P. allocasuarinae' | '*Candidatus* Phytoplasma allocasuarinae' | 16SrX | AY135523 |
| '*Ca.* P. rhamni' | '*Candidatus* Phytoplasma rhamni' | 16SrX | X76431 |
| *C. dactylon* | *Cynodon dactylon* phytoplasma | **16SrXVI** | AY742327 |
| SCYLP | YLS phytoplasma | **16SrXVI** | AY725228 |
| *C. canadensis* | *Conyza canadensis* phytoplasma | **16SrXVI** | AY742328 |
| *M. lathyroides* | *Macroptilium lathyroides* phytoplasma | **16SrXVI** | AY742329 |
| *S. saccharivora* | *Saccharosydne saccharivora* phytoplasma | **16SrXVI** | AY725229 |
| *S. halepense* | *Sorghum halepense* phytoplasma | **16SrXVI** | AY742330 |
| DP | *Cedusa* derbid phytoplasma | **16SrXVI** | AY744944 |
| *Emp3* | *Empoasca* phytoplasma, sample 3 | **16SrXVII** | AY725235 |
| PAY | Papaya phytoplasma | **16SrXVII** | AY725234 |
| VK | *Vitis vinifera* phytoplasma | 16SrXII | X76428 |
| STOLS | *Capsicum anuum* to *Catharanthus roseus* phytoplasma | 16SrXII | X76427 |
| STOL | Stolbur | 16SrXII | AF248959 |
| PYL2 | *Phormium* yellow leaf | 16SrXII | U43570 |

**Table 1.** cont.

| Acronym | Phytoplasma strain designation | RFLP group | Accession number |
|---|---|---|---|
| '*Ca.* P. australiense' | '*Candidatus* Phytoplasma australiense' | 16SrXII | L76865 |
| SGP | Strawberry green petal | 16SrXII | AJ243044 |
| SLY | Strawberry lethal yellows | 16SrXII | AJ243045 |
| PDB | Papaya dieback | 16SrXII | Y10095 |
| PYL1 | *Phormium* yellow leaf | 16SrXII | U43569 |
| '*Ca.* P. japonicum' | '*Candidatus* Phytoplasma japonicum' | 16SrI | AB010425 |
| MPV | Periwinkle virescence | 16SrXIII | AF248960 |
| AAY | American aster yellows | 16SrI | X68373 |
| '*Ca.* P. asteris' | '*Candidatus* Phytoplasma asteris' | 16SrI | M30790 |
| *A. palmae* | *Acholeplasma palmae* | – | L33734 |
| *A. laidlawii* | *Acholeplasma laidlawii* | – | M23932 |

**Table 2.** Results of nPCR from sugarcane, papaya, weeds, *Saccharosydne saccharivora*, *Cedusa* spp. and *E. papayae*

| Plant/insect species | Plants/insects analysed (*n*) | Positive nPCR/*Hae*III results (*n*) |
|---|---|---|
| Sugarcane | 120 | 102/17* |
| Papaya | 106 | 93/11* |
| Weeds | 50 | 11/18* |
| *Saccharosydne saccharivora* | 21 | 21 |
| *Cedusa* spp. | 19 | 16 |
| *E. papayae* | 23 | 11 |

*With symptoms/without symptoms.

amplifications were not obtained with the DNA of any papaya or *E. papayae* using the primer pair PBTF1/PBTR1.

## RFLP analysis and putative restriction sites in phytoplasma rRNA operon sequences

Results obtained after incubation with restriction endonucleases and electrophoresis (Figs 1, 2 and 3) indicated that, although some phytoplasmas associated with sugarcane, papaya, weeds, *Saccharosydne saccharivora*, *Cedusa* spp. and *E. papayae* showed slight differences among their 16S rRNA gene RFLP patterns, when compared with the reference phytoplasmas, they could be distinguished from each other and from the rest of the strains analysed. On the other hand, their putative rRNA operon sequence restriction-site maps showed contrasting results (Fig. 4), when compared with those of the other phytoplasma groups analysed.

The uppermost bands of the *Alu*I profiles of the unknown phytoplasmas showed a slight difference in size compared with those of known phytoplasmas (Fig. 1), which might explain why putative restriction-site analysis of their 16S rRNA gene sequences showed an additional *Alu*I site (Fig. 4) that was absent from the other known phytoplasma groups analysed.

The *Tru*9I RFLP profiles of the 16S rRNA gene of phytoplasmas identified in sugarcane, weeds and *Saccharosydne saccharivora* were similar to those of Stolbur and AAY reference strains (Fig. 1). However, restriction profiles of *Cedusa* spp., papaya and *E. papayae* phytoplasmas had an additional band at 600 bp, which was present in AAY and ACLR reference phytoplasmas, but not in the 16S rRNA genes of phytoplasmas of sugarcane, *Saccharosydne*

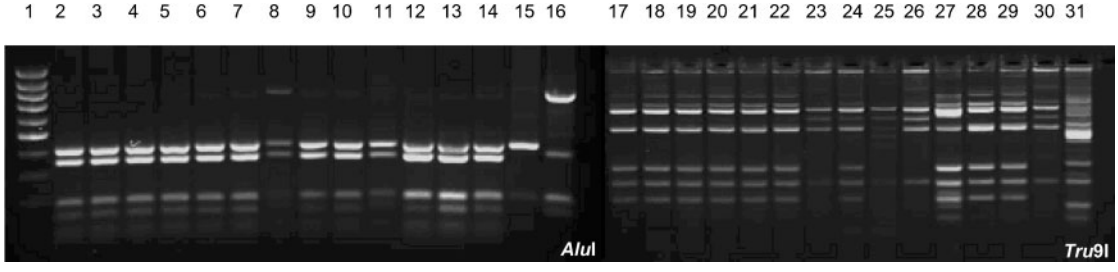


**Fig. 1.** RFLP analysis of 16S rRNA genes amplified by nPCR with *Alu*I and *Tru*9I enzymes. Lanes: 1, 100 bp ladder (MBI Fermentas); 2 and 17, SCYLP; 3 and 18, *Saccharosydne saccharivora*; 4 and 19, *Cynodon dactylon*; 5 and 20, *Conyza canadensis*; 6 and 21, *Macroptilium lathyroides*; 7 and 22, *Sorghum halepense*; 8 and 23, *Cedusa* spp.; 9 and 24, PAY; 10 and 25, *Emp3*; 11 and 26, *Emp5*; 12 and 27, STOL; 13 and 28, VK; 14 and 29, AAY; 15 and 30, ACLR; 16 and 31, PLN.

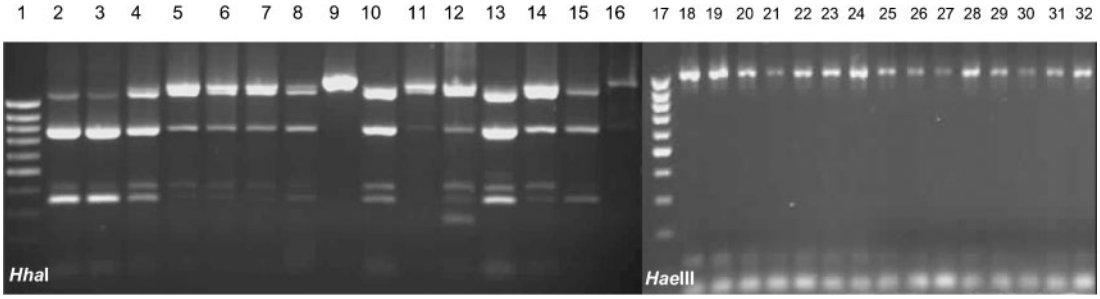


**Fig. 2.** RFLP analysis of 16S rRNA genes amplified by nPCR with *Hha*I and *Hae*III enzymes. Lanes: 1 and 17, 100 bp ladder (MBI Fermentas); 2 and 18, SCYLP; 3 and 19, *Saccharosydne saccharivora*; 4 and 20, *Cynodon dactylon*; 5 and 21, *Conyza canadensis*; 6 and 22, *Macroptilium lathyroides*; 7 and 23, *Sorghum halepense*; 8 and 24, *Cedusa* spp.; 9 and 25, PAY; 10 and 26, *Emp3*; 11 and 27, *Emp5*; 12 and 28, STOL; 13 and 29, VK; 14 and 30, AAY; 15 and 31, ACLR; 16 and 32, PLN.

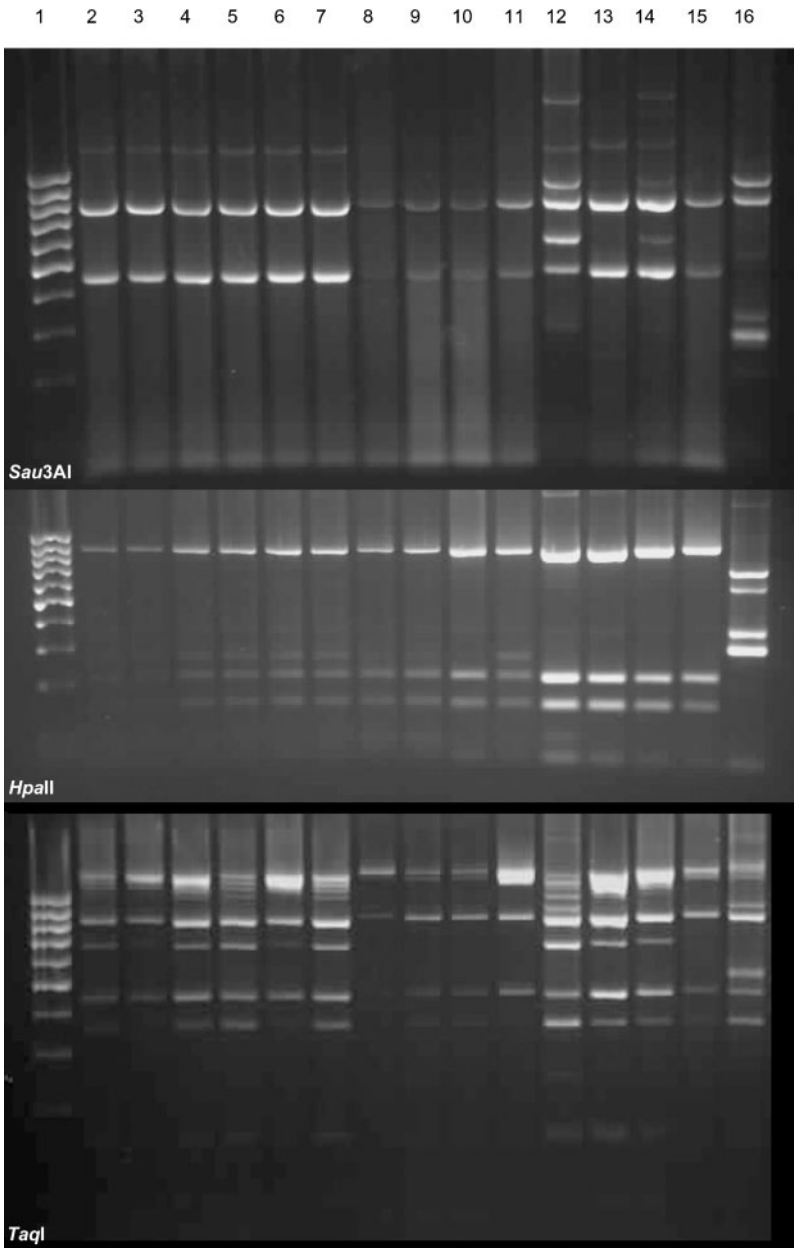


**Fig. 3.** RFLP analysis of 16S rRNA genes amplified by nPCR with *Sau*3AI, *Hpa*II and *Taq*I enzymes. Lanes: 1, 100 bp ladder (MBI Fermentas); 2, SCYLP; 3, *Saccharosydne saccharivora*; 4, *Cynodon dactylon*; 5, *Conyza canadensis*; 6, *Macroptilium lathyroides*; 7, *Sorghum halepense*; 8, *Cedusa* spp.; 9, PAY; 10, *Emp3*; 11, *Emp5*; 12, STOL; 13, VK; 14, AAY; 15, ACLR; 16, PLN.

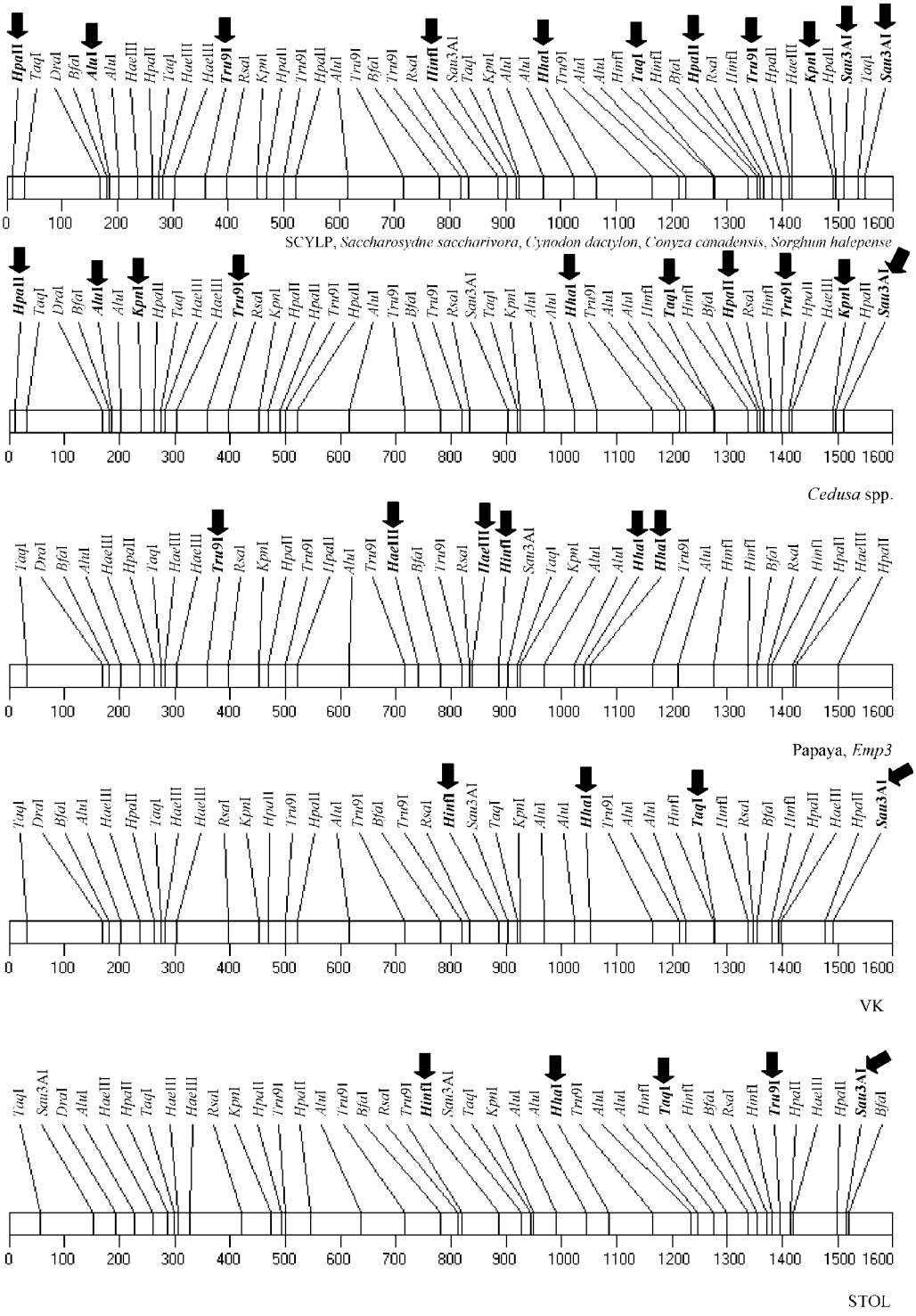


**Fig. 4.** Analysis of putative restriction sites of phytoplasma 16S rRNA gene sequences. Maps were generated by using the RESearch program of the Invitro package (Rothamsted Research) and were manually aligned for comparison of recognition sites for restriction endonucleases *Alu*I, *Bfa*I, *Dra*I, *Hpa*II, *Hha*I, *Hin*fI, *Kpn*I, *Rsa*I, *Sau*3AI, *Taq*I, *Hae*III and *Tru*9I. Arrows indicate sites that are different between the Cuban phytoplasmas and the two reference strains analysed, VK (GenBank accession no. X76428) and STOL (AF248959).

*saccharivora* and weeds. Putative restriction analysis (Fig. 4) revealed a *Tru*9I site in the 16S rRNA genes of sugarcane, *Saccharosydne saccharivora* and weed phytoplasmas, which was also present in the 16S rRNA gene sequence of the Stolbur reference phytoplasma, but not in those of papaya and *E. papayae*. Moreover, there were other additional *Tru*9I sites that distinguished phytoplasmas in sugarcane, *Saccharosydne saccharivora*, weeds, *Cedusa* spp., papaya and *E. papayae* from the reference strains they were compared with.

The *Sau*3AI restriction profiles (Fig. 3) of phytoplasmas in sugarcane, weeds and *Saccharosydne saccharivora* were similar to that of the VK reference phytoplasma strain; however, we noted that all the bands of the VK strain profile were slightly larger in size than those of the unknown phytoplasmas. In addition, the *Sau*3AI RFLP patterns of phytoplasmas identified in papaya, *Cedusa* spp. and *E. papayae* were more similar to that of the ACLR reference phytoplasma, but the latter also showed a band that was slightly larger in size. From putative restriction-map comparisons (Fig. 4), all of the 16S rRNA gene sequences of the phytoplasmas analysed shared a *Sau*3AI site at the position specified by the arrows, except for the papaya and *E. papayae* phytoplasmas; this site is characteristic of the latter phytoplasmas, and could explain their RFLP profiles. However, differentiation could also be justified by the presence of an additional *Sau*3AI site in the 16S rRNA gene sequences of sugarcane, *Saccharosydne saccharivora* and weed phytoplasmas, which distinguished them from the other phytoplasmas analysed.

The *Hpa*II 16S rRNA gene RFLP patterns (Fig. 3) of phytoplasmas in papaya and *E. papayae* were similar to those of the AAY and ACLR reference strains. However, in the case of the sugarcane, *Saccharosydne saccharivora*, weeds and *Cedusa* spp. 16S rRNA gene profiles, a band of approximately 270 bp distinguished them from the other phytoplasmas analysed. This might be supported by the putative restriction analysis (Fig. 4), as their 16S rRNA gene sequences showed additional *Hpa*II sites that were not present in those of the other phytoplasmas mapped.

The *Taq*I 16S rRNA gene RFLP patterns of phytoplasmas (Fig. 3) in sugarcane, weeds and *Saccharosydne saccharivora*, which were similar to that of the VK reference strain, showed that the bottom three bands of the profiles were slightly smaller in size, which distinguished them from VK and the other phytoplasmas analysed. However, the *Taq*I 16S rRNA gene RFLP profiles of phytoplasmas in *Cedusa* spp., papaya and *E. papayae* were similar to that of the ACLR reference strain. Putative restriction analysis (Fig. 4) revealed the lack of a *Taq*I site in the 16S rRNA gene sequences of papaya and *E. papayae* phytoplasmas, and the displacement of this site in the 16S rRNA gene sequences of phytoplasmas in sugarcane, weeds and *Saccharosydne saccharivora*, when compared with those of VK and Stolbur reference strains, which might explain the slight differences in their RFLP patterns.

The *Hha*I 16S rRNA gene RFLP patterns of phytoplasmas in sugarcane and *Saccharosydne saccharivora* were similar to that of the VK reference strain (Fig. 2), whereas those of the other phytoplasmas were similar to the AAY *Hha*I profile. From putative restriction-map comparisons (Fig. 4), the 16S rRNA gene sequences of all the phytoplasmas analysed shared one *Hha*I site at the positions specified by the arrows; however, an additional *Hha*I site distinguished phytoplasmas identified in papaya and *E. papayae* from the other strains analysed.

No differences were found among the *Hae*III 16S rRNA gene RFLP patterns of phytoplasmas analysed (Fig. 2). According to the putative restriction-map analysis (Fig. 4), the 16S rRNA gene sequences of phytoplasmas in papaya and *E. papayae* only showed two additional *Hae*III sites, which distinguished them from the other strains mapped.

Putative restriction analysis (Fig. 4) also revealed that phytoplasmas in sugarcane, weeds, *Saccharosydne saccharivora* and *Cedusa* spp. could be distinguished from the other strains analysed in that their 16S rRNA gene sequences showed an additional *Kpn*I site, which was not present in the 16S rRNA gene of all other 16SrXII phytoplasmas mapped.

The 16S rRNA gene sequence of the phytoplasma identified in *Cedusa* spp. had an additional *Hpa*II site and lacked a *Hin*fI site, which are the distinguishing characteristics of this phytoplasma.

## Sequence similarity

The 16S rRNA gene sequences of phytoplasmas from sugarcane and *Saccharosydne saccharivora* planthoppers were identical (100 % similarity), and shared 99 % similarity with the 16S rRNA genes amplified from *Cynodon dactylon*, *Sorghum halepense*, *Conyza canadensis* and *Macroptilium lathyroides*, and 98 % similarity with the 16S rRNA gene sequence from *Cedusa* spp. planthopper (DP). The phytoplasma 16S rRNA gene sequences from papaya (PAY) and an *E. papayae* sample (*Emp3*) also showed 100 % similarity, whereas the sequence similarities were 95 % when compared with the 16S rRNA genes from sugarcane, *Saccharosydne saccharivora* and weeds and 95·5 % when compared with the 16S rRNA gene from *Cedusa* spp.

The sequence similarities of the phytoplasma 16S rRNA genes from sugarcane, *Saccharosydne saccharivora*, *Cynodon dactylon*, *Sorghum halepense*, *Conyza canadensis* and *Macroptilium lathyroides* were 97·5 % to those of previously characterized phytoplasma strains STOL, VK and *Phormium* yellow leaf (PYL1); 97 % to *Capsicum anuum* to *Catharanthus roseus* phytoplasma (STOLS); 95 % to PYL2, '*Candidatus* Phytoplasma australiense', Papaya dieback (PDB), Strawberry green petal (SGP) and Strawberry lethal yellows (SLY); 94·5 % to '*Candidatus* Phytoplasma japonicum'; and 93·5 % to AAY and '*Candidatus* Phytoplasma asteris'. The similarity of the amplified 16S rRNA gene from *Cedusa* spp. was 96 % to those of STOL, VK and STOLS; 94·5 % to PYL1, PYL2, '*Ca.*

P. australiense', PDB and SGP; 93·5 % to SLY; and 93 % to Periwinkle virescence (MPV), '*Ca.* P. japonicum', AAY and '*Ca.* P. asteris'. Similarities to other known phytoplasma group representatives ranged from 86 to 89·7 %.

The sequence similarities of 16S rRNA genes amplified from PAY and *Emp3* phytoplasmas were 97 % to those of STOL, VK and STOLS; 95·5 % to '*Ca.* P. japonicum'; 95 % to PYL1, PYL2, '*Ca.* P. australiense', PDB, SGP and SLY; and 93·5 % to MPV, AAY and '*Ca.* P. asteris'. Similarities to other phytoplasma groups ranged from 86 to 89·7 %.

The 16S rRNA gene sequence of the phytoplasma identified in *Empoasca* sample 5 (*Emp5*) was 98 % similar to that of '*Candidatus* Phytoplasma allocasuarinae'; 93 % to Pear decline (PD), '*Candidatus* Phytoplasma pyri', '*Candidatus* Phytoplasma prunorum' and '*Candidatus* Phytoplasma rhamni'; 92 % to Apple proliferation (AP) and '*Candidatus* Phytoplasma mali'; 90 % to representatives from groups 16SrI, 16SrII, 16SrVI, 16SrXI, 16SrXIV and 16SrXV; 89 % to representatives from groups 16SrIII, 16SrIV, 16SrV, 16SrVII, 16SrVIII, 16SrIX, 16SrXII, 16SrXIII, papaya and *Emp3*; and 88·5 % to phytoplasmas in sugarcane, *Saccharosydne saccharivora*, weeds and *Cedusa* spp.

## Similarity coefficients

Similarity coefficients derived from RFLP analysis were calculated on the basis of putative restriction-site analysis of nucleotide sequences of 16S rRNA genes (Table 3). The unknown phytoplasma sequences were compared with those of 16S rRNA gene phytoplasma groups included in the analysis of sequence similarity. This analysis revealed that members of the same 16S rRNA gene RFLP group shared values that were $>0{\cdot}82$. Stolbur (16SrXII) phytoplasmas shared values ranging from 0·83 (PYL2 with STOLS) to 0·98 (SLY with SGP), whereas RFLP similarity coefficients between phytoplasmas from the Aster yellows group (16SrI) reached 0·99 ('*Ca.* P. asteris' with AAY). The 16S rRNA gene similarity coefficients between phytoplasmas in sugarcane, *Saccharosydne saccharivora*, *Macroptilium lathyroides*, *Conyza canadensis*, *Sorghum halepense*, *Cynodon dactylon* and *Cedusa* spp. ranged from 0·82 (PAY and *Emp3* with *Cedusa* spp.) to 0·99 [YLS phytoplasma (SCYLP) and *Saccharosydne saccharivora* with *Sorghum halepense* and *Conyza canadensis*, as well as *Sorghum halepense* and *Macroptilium lathyroides* with *Cynodon dactylon*].

From comparisons among members belonging to different groups, phytoplasmas from the 16SrXII group exhibited a range from 0·56 (MPV with STOLS) to 0·69 (MPV with '*Ca.* P. australiense'), when compared with members of the 16SrXIII group; from 0·59 ('*Ca.* P. allocasuarinae' with PYL2) to 0·76 ('*Ca.* P. allocasuarinae' with STOL); and from 0·62 (AP with STOLS) to 0·76 (AP with STOL), when compared with members of the 16SrX group. Comparisons of similarity coefficients between phytoplasmas from the 16SrI and 16SrX groups showed values of 0·71 (AAY with AP), 0·77 (AAY with '*Ca.* P. allocasuarinae'), 0·73 ('*Ca.* P. asteris' with '*Ca.* P. allocasuarinae') and 0·67 ('*Ca* P. asteris' with AP).

The 16S rRNA gene similarity coefficients of unknown phytoplasmas identified in Cuba ranged from 0·78 (*Cedusa* spp. with PYL1) to 0·94 (SCYLP and *Saccharosydne saccharivora* with VK and STOLS), when compared with phytoplasmas from the 16SrXII group; from 0·5 (*Cynodon dactylon*) to 0·82 (SCYLP and *Saccharosydne saccharivora*), when compared with MPV from the 16SrXIII group; from 0·6 (*Sorghum halepense* with '*Ca.* P. asteris') to 0·88 (PAY and *Emp3* with AAY), when compared with the 16SrI group; and from 0·42 (*Sorghum halepense* with '*Ca.* P. allocasuarinae') to 0·75 (SCYLP and *Saccharosydne saccharivora* with AP), when compared with the 16SrX group.

These results clearly distinguished phytoplasmas in sugarcane, weeds, *Saccharosydne saccharivora* and *Cedusa* spp. from those present in papaya and *Emp3* and from the reference strains analysed. The findings support recognition of the phytoplasmas present in sugarcane, *Saccharosydne saccharivora*, *Macroptilium lathyroides*, *Conyza canadensis*, *Sorghum halepense*, *Cynodon dactylon* and *Cedusa* spp., and those from papaya and *Emp3*, as representing two new 16S rRNA gene RFLP groups.

## Phylogenetic analysis

Phylogenetic analysis of 62 phytoplasmas, *Acholeplasma palmae* and *Acholeplasma laidlawii* produced the consensus tree illustrated in Fig. 5. Bootstrapping values strongly supported most branches, indicating a robust tree whose branching order is in good agreement with previous findings (Lee *et al.*, 1998; White *et al.*, 1998; Jung *et al.*, 2003, 2004). However, phytoplasmas in Cuban sugarcane, weeds, *Saccharosydne saccharivora* and the *Cedusa* spp. gave rise to a new branch, whereas phytoplasmas in papaya and *Emp3* formed a second new branch, compared with previously published phylogenetic trees in which 15 phytoplasma subclades were identified (IRPCM Phytoplasma/Spiroplasma Working Team–Phytoplasma Taxonomy Group, 2004). Although these two new branches are closely related to the 16SrXII Stolbur group, according to the species definition of Stackebrandt & Goebel (1994) and the classification system of Lee *et al.* (1998), differences in 16S rRNA gene RFLP patterns and sequence similarities show that the phytoplasmas from sugarcane, weeds, *Saccharosydne saccharivora* and *Cedusa* spp., and those from papaya and *Emp3*, represent two new lineages (16SrXVI and 16SrXVII, respectively) that are distinct from 16SrXII (Stolbur) and all other phytoplasma groups.

## Sequences unique to phytoplasmas in the 16S rRNA gene of the novel phytoplasmas identified

The 16S rRNA gene sequences from phytoplasmas associated with sugarcane, weeds, papaya, *Saccharosydne saccharivora* and the *Cedusa* planthopper were aligned with sequences from 54 phytoplasmas representing the 15 current

**Table 3.** Similarity coefficients derived from RFLPs based on putative restriction-site analysis of nucleotide sequences of phytoplasma 16S rRNA genes of sugarcane, weeds, *Saccharosydne saccharivora*, *Cedusa* spp., papaya, *Emp*3 and other selected phytoplasmas

See Table 1 for abbreviations of phytoplasmas.

| Phytoplasma | 1 | 2 | 3 | 4 | 5 | 6 | 7 | 8 | 9 | 10 | 11 | 12 | 13 | 14 | 15 | 16 | 17 | 18 | 19 | 20 | 21 | 22 | 23 | 24 | 25 |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| 1. SCYLP | 1 | 1 | 0·97 | 0·99 | 0·99 | 0·98 | 0·97 | 0·92 | 0·92 | 0·72 | 0·94 | 0·94 | 0·91 | 0·91 | 0·87 | 0·87 | 0·89 | 0·89 | 0·88 | 0·79 | 0·82 | 0·86 | 0·81 | 0·71 | 0·75 |
| 2. *S. saccharivora* | | 1 | 0·97 | 0·99 | 0·99 | 0·98 | 0·97 | 0·92 | 0·92 | 0·72 | 0·94 | 0·94 | 0·91 | 0·91 | 0·87 | 0·87 | 0·89 | 0·89 | 0·88 | 0·79 | 0·82 | 0·86 | 0·81 | 0·71 | 0·75 |
| 3. *M. lathyroides* | | | 1 | 1 | 1 | 0·99 | 0·96 | 0·86 | 0·86 | 0·67 | 0·93 | 0·95 | 0·89 | 0·86 | 0·91 | 0·87 | 0·88 | 0·87 | 0·83 | 0·75 | 0·63 | 0·83 | 0·77 | 0·69 | 0·66 |
| 4. *S. halepense* | | | | 1 | 1 | 0·99 | 0·96 | 0·85 | 0·85 | 0·69 | 0·88 | 0·85 | 0·85 | 0·88 | 0·88 | 0·87 | 0·88 | 0·87 | 0·84 | 0·64 | 0·68 | 0·87 | 0·6 | 0·42 | 0·45 |
| 5. *C. canadensis* | | | | | 1 | 0·99 | 0·96 | 0·89 | 0·89 | 0·69 | 0·89 | 0·95 | 0·89 | 0·86 | 0·88 | 0·87 | 0·88 | 0·87 | 0·82 | 0·75 | 0·67 | 0·81 | 0·82 | 0·69 | 0·66 |
| 6. *C. dactylon* | | | | | | 1 | 0·95 | 0·88 | 0·88 | 0·65 | 0·9 | 0·88 | 0·87 | 0·85 | 0·86 | 0·85 | 0·86 | 0·84 | 0·8 | 0·74 | 0·5 | 0·81 | 0·8 | 0·67 | 0·65 |
| 7. *Cedusa* spp. | | | | | | | 1 | 0·82 | 0·82 | 0·61 | 0·87 | 0·87 | 0·85 | 0·82 | 0·79 | 0·85 | 0·86 | 0·83 | 0·78 | 0·71 | 0·62 | 0·72 | 0·71 | 0·63 | 0·62 |
| 8. *Emp3* | | | | | | | | 1 | 1 | 0·8 | 0·89 | 0·83 | 0·85 | 0·87 | 0·92 | 0·9 | 0·92 | 0·86 | 0·79 | 0·81 | 0·68 | 0·88 | 0·85 | 0·73 | 0·64 |
| 9. PAY | | | | | | | | | 1 | 0·8 | 0·89 | 0·83 | 0·85 | 0·87 | 0·92 | 0·9 | 0·92 | 0·86 | 0·79 | 0·81 | 0·68 | 0·88 | 0·85 | 0·73 | 0·64 |
| 10. *Emp5* | | | | | | | | | | 1 | 0·5 | 0·52 | 0·5 | 0·47 | 0·57 | 0·52 | 0·53 | 0·42 | 0·46 | 0·62 | 0·61 | 0·64 | 0·55 | 0·85 | 0·65 |
| 11. VK | | | | | | | | | | | 1 | 0·9 | 0·96 | 0·89 | 0·96 | 0·9 | 0·92 | 0·9 | 0·89 | 0·81 | 0·65 | 0·88 | 0·96 | 0·73 | 0·78 |
| 12. STOLS | | | | | | | | | | | | 1 | 0·87 | 0·83 | 0·92 | 0·85 | 0·86 | 0·84 | 0·83 | 0·79 | 0·56 | 0·86 | 0·85 | 0·61 | 0·62 |
| 13. STOL | | | | | | | | | | | | | 1 | 0·89 | 0·96 | 0·9 | 0·92 | 0·94 | 0·89 | 0·85 | 0·68 | 0·92 | 0·91 | 0·76 | 0·76 |
| 14. PYL2 | | | | | | | | | | | | | | 1 | 0·85 | 0·91 | 0·93 | 0·95 | 0·93 | 0·73 | 0·61 | 0·81 | 0·8 | 0·59 | 0·79 |
| 15. '*Ca.* P. australiense' | | | | | | | | | | | | | | | 1 | 0·86 | 0·88 | 0·86 | 0·86 | 0·85 | 0·69 | 0·92 | 0·91 | 0·74 | 0·77 |
| 16. SGP | | | | | | | | | | | | | | | | 1 | 0·98 | 0·96 | 0·9 | 0·85 | 0·67 | 0·9 | 0·89 | 0·65 | 0·67 |
| 17. SLY | | | | | | | | | | | | | | | | | 1 | 0·9 | 0·9 | 0·82 | 0·62 | 0·9 | 0·87 | 0·67 | 0·67 |
| 18. PDB | | | | | | | | | | | | | | | | | | 1 | 0·9 | 0·82 | 0·65 | 0·86 | 0·85 | 0·64 | 0·71 |
| 19. PYL1 | | | | | | | | | | | | | | | | | | | 1 | 0·83 | 0·67 | 0·86 | 0·87 | 0·69 | 0·8 |
| 20. '*Ca.* P. japonicum' | | | | | | | | | | | | | | | | | | | | 1 | 0·59 | 0·97 | 1 | 0·75 | 0·67 |
| 21 MPV | | | | | | | | | | | | | | | | | | | | | 1 | 0·82 | 0·81 | 0·74 | 0·78 |
| 22. AAY | | | | | | | | | | | | | | | | | | | | | | 1 | 0·99 | 0·77 | 0·71 |
| 23. '*Ca.* P. asteris' | | | | | | | | | | | | | | | | | | | | | | | 1 | 0·73 | 0·67 |
| 24. '*Ca.* P. allocasuarinae' | | | | | | | | | | | | | | | | | | | | | | | | 1 | 0·65 |
| 25. AP | | | | | | | | | | | | | | | | | | | | | | | | | 1 |

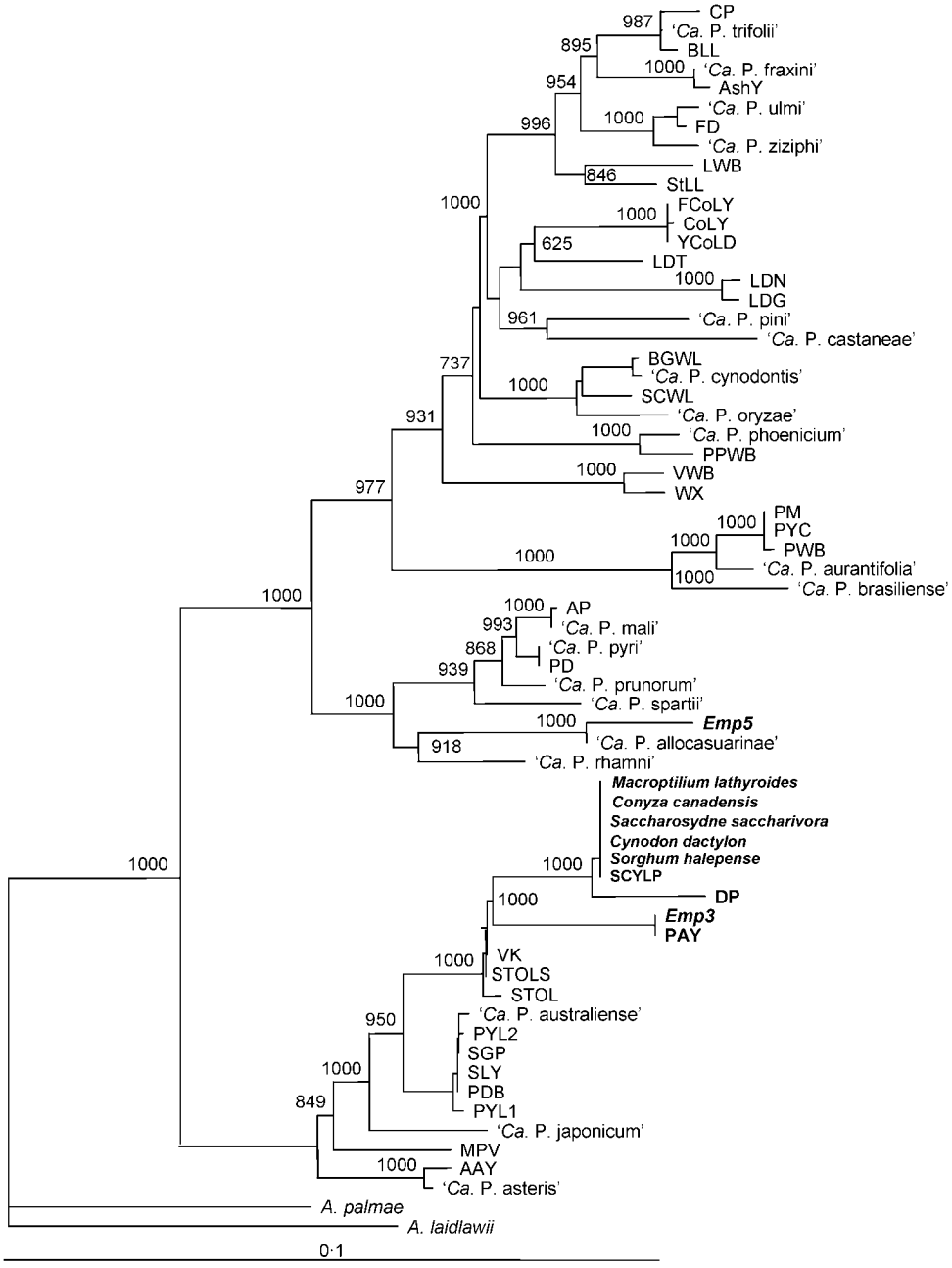


**Fig. 5.** Phylogenetic tree of 16S rRNA gene sequences constructed by parsimony analysis showing the relationships between phytoplasmas detected in sugarcane, *Saccharosydne saccharivora*, *Cynodon dactylon*, *Conyza canadensis*, *Macroptilium lathyroides*, *Sorghum halepense*, *Cedusa* spp., papaya and *E. papayae* (samples 3 and 5) and the other GenBank reference phytoplasmas analysed. Numbers above branches are bootstrap values obtained for 1000 replicates. Branch lengths are proportional to the number of inferred character state transformations. *Acholeplasma laidlawii* was used as the outgroup. See Table 1 for abbreviations of phytoplasmas and GenBank accession numbers. Bar, phylogenetic distance of 10 %.

phytoplasma groupings (IRPCM Phytoplasma/Spiroplasma Working Team–Phytoplasma Taxonomy Group, 2004). This analysis revealed that these phytoplasmas contain sequences unique to phytoplasmas and sequences that also distinguish them from other known phytoplasmas.

Sequences previously reported to be unique to phytoplasmas (Gundersen *et al.*, 1994; Davis *et al.*, 1997), 5′-TTTTAAAAG-3′ at positions 167–175, 5′-GTGT-3′ at positions 256–259, 5′-TGGAGG-3′ at positions 347–352, 5′-GGCAAG-3′ at positions 632–637, 5′-ATCAG-3′ at

positions 992–996, 5′-TAGC-3′ at positions 1212–1215 and 5′-AGTT-3′ at positions 1290–1293, were present in the 16S rRNA gene of the novel phytoplasmas identified. The other signature sequence 5′-ACTGGA-3′ also occurred at positions 135–140 but, in the case of the *Cedusa* spp. phytoplasma, was replaced by TCTGGA.

Unique sequences from the 16S rRNA genes of the phytoplasmas from sugarcane, weeds, *Saccharosydne saccharivora* and *Cedusa* derbid were 5′-TTTG-3′ at positions 424–427, 5′-TTG-3′ at positions 436–439, 5′-GGG-3′ at positions 1490–1492, 5′-TAA-3′ at positions 1326–1328 and 5′-ATTTACGTTTCTG-3′ at positions 1330–1342. These differ at all positions from corresponding sequences from phytoplasmas in other subclades. The *Cedusa* sp. phytoplasma was the only one to contain the unique sequence 5′-CCC-3′ at positions 508–510 with respect to the 16S rRNA gene of the other phytoplasmas analysed, including those identified in Cuba.

The 16S rRNA gene of the phytoplasma amplified from both papaya and *Emp3* had the following signature sequences: 5′-AAA-3′ at positions 161–163, 5′-ATT-3′ at positions 558–560, 5′-AGGCGCC-3′ at positions 1039–1045 and 5′-GCGGATTTAGTCACTTTTCAGGC-3′ at positions 1324–1346, which differ from those of all other known phytoplasmas at all positions.

## DISCUSSION

Amplification of characteristic phytoplasma rRNA gene fragments with the typical *Hae*III phytoplasma profile (Aljanabi *et al.*, 2001) was obtained from diseased sugarcane, weeds and papaya and from putative leafhopper vectors *Saccharosydne saccharivora*, *Cedusa* spp. and *E. papayae*. The presence of sequences unique to phytoplasmas from both papaya and *E. papayae* DNA and the absence of any rickettsial amplimers establish that the organisms associated with sugarcane YLS, papaya PBT-like disease and those present in weeds and putative vectors are phytoplasmas.

Phytoplasmas were detected in *Saccharosydne saccharivora* collected from YLS-infected sugarcane fields, supporting its role as the vector of the phytoplasma associated with YLS (Arocha *et al.*, 2005b). Leafhoppers and planthoppers are the most prolific natural vectors of phytoplasma diseases worldwide (Fletcher *et al.*, 1998; Carraro *et al.*, 2001), so the detection and identification of the phytoplasma present in *Cedusa* spp. suggests that they might be a putative vector of YLS and should be a target for future transmission studies.

Although leafhopper vectors of phytoplasmas infecting papaya are unknown, a species of *Orosius* has been identified as a candidate for transmission studies of papaya diseases in Australia (Padovan & Gibb, 2001). The leafhopper *E. papayae* has been reported to be a natural vector of PBT disease (Davis *et al.*, 1998); however, from our study, all *E. papayae* captured from the papaya plantations showing PBT-like symptoms were negative when indexed by PCR using the specific rickettsial primer pair PBTF1/PBTR1, whereas 104 of 106 plants tested clearly showed 16S rRNA gene signatures. This strongly indicates that phytoplasmas are consistently associated with the disease in Cuba, playing a fundamental role in its development, and that *E. papayae* is a putative vector.

*Cynodon dactylon* and *Conyza canadensis*, which tested positive for phytoplasma, displayed typical white leaf and witches'-broom symptoms, respectively. Symptoms of white leaf *Cynodon dactylon* plants have been previously associated with the *Cynodon* white leaf phytoplasma (Arocha *et al.*, 2005a). However, although *Macroptilium lathyroides* and *Sorghum halepense* tested positive for phytoplasma, they were asymptomatic, suggesting that weeds present in and surrounding sugarcane and papaya plantations serve as phytoplasma reservoirs. Further studies will be required to determine the various factors involved in their roles in the epidemiology of YLS and PBT-like diseases.

Symptomless phytoplasma infections in sugarcane occur widely (Bailey *et al.*, 1996; Cronjé *et al.*, 1998; Arocha *et al.*, 2000; Tran-Nguyen *et al.*, 2000; Aljanabi *et al.*, 2001), and the relatively long growth period of this crop allows infections to be carried through seasonal barriers and crop cycles. In this study, 14·2 % of sugarcane plants without symptoms were infected with phytoplasmas. Similarly, 10·6 % of symptomless papaya plants contained phytoplasmas, demonstrating that latent infections can also occur in papaya.

RFLP analysis has been found to be useful for general classification (Schneider *et al.*, 1993; Lee *et al.*, 1998; Seemüller *et al.*, 1998) although, in some cases, phytoplasma groups classified on the basis of this method were not always consistent with phylogenetic grouping (Lee *et al.*, 1998). However, RFLP analysis has proved to be a simple and rapid tool for the preliminary classification and identification of unknown phytoplasmas in a relatively short time (Lee *et al.*, 1998; Seemüller *et al.*, 1998). *Hae*III and *Hha*I 16S rRNA gene RFLP patterns obtained from our study could not distinguish the unknown phytoplasmas from reference phytoplasmas; however, *Alu*I, *Sau*3AI, *Hpa*II and *Taq*I enzymes yielded restriction profiles that could differentiate phytoplasmas in sugarcane, weeds and *Saccharosydne saccharivora* as a distinct 16S rRNA gene RFLP group from those in papaya and *E. papayae* (*Emp3*) and the other phytoplasmas analysed. This was also supported by clear differences in putative 16S rRNA gene restriction maps, including similarity coefficient calculations and sequence similarity analysis.

The International Committee on Systematics of Prokaryotes Subcommittee on the Taxonomy of *Mollicutes* has recommended the inclusion of 16S rRNA gene sequences in any description of a novel mollicute species (Marcone *et al.*, 2004a, b). According to Stackebrandt & Goebel (1994), at sequence similarity values below about 97·5 % (in the 16S rRNA gene), it is unlikely that two organisms have more than 60 to 70 % DNA relatedness and hence that they are

related at the species level. For uncultured phytoplasmas, a novel putative species may be described when its 16S rRNA gene sequence (1200 bp) has ⩽97·5 % similarity to any previously described '*Candidatus* Phytoplasma' species (IRPCM Phytoplasma/Spiroplasma Working Team–Phytoplasma Taxonomy Group, 2004).

A mean sequence similarity of 95·58 % between phytoplasmas in the 16SrXII Stolbur group and those detected in sugarcane, *Saccharosydne saccharivora*, *Cynodon dactylon*, *Sorghum halepense*, *Conyza canadensis*, *Macroptilium lathyroides* and *Cedusa* spp. was demonstrated in this study, whereas similarities between the latter strains and PAY or *Emp3* phytoplasmas are slightly greater, at 95·8 %. The unknown phytoplasmas also showed 85–93 % similarity in their 16S rRNA gene sequences compared with representatives of other established phytoplasma groups. Although they are most closely related to phytoplasmas of the Stolbur 16SrXII group, all data presented here and the presence of signature sequences in their respective 16S rRNA genes demonstrate that these phytoplasmas represent two novel provisional species. This conclusion is supported by differences in 16S rRNA gene RFLP patterns and putative restriction maps of unknown phytoplasmas when compared with the reference groups analysed, which, together with the phylogenetic analysis, have demonstrated these phytoplasmas to be representatives of two new 16S rRNA gene RFLP groups (16SrXVI and 16SrXVII, respectively), which are distinct from other phytoplasma groups.

We propose that these phytoplasmas should be given *Candidatus* status, according to the scheme for assigning incompletely described prokaryotes to the provisional status '*Candidatus*', implemented by the International Committee on Systematic Bacteriology (Murray & Stackebrandt, 1995). We propose that the phytoplasma in sugarcane in Cuba should be designated '*Candidatus* Phytoplasma graminis' and that strains identified in *Saccharosydne saccharivora*, *Cedusa* spp., *Cynodon dactylon*, *Conyza canadensis*, *Macroptilium lathyroides* and *Sorghum halepense* should be considered to be related to this novel species. For the phytoplasma in papaya, including the related strain in *E. papayae*, we propose the name '*Candidatus* Phytoplasma caricae', with the following descriptions.

'*Candidatus* Phytoplasma graminis' (L. gen. n. *graminis* of grass, herb; epithet referring to the plant host) [(*Mollicutes*) NC; NA; O, wall-less; NAS (GenBank accession no. AY725228); oligonucleotide sequences of unique regions of 16S rRNA gene: 5′-TTTG-3′, 5′-TTG-3′, 5′-GGG-3′, 5′-TAA-3′ and 5′-ATTTACGTTTCTG-3′; P (*Saccharum officinarum*; phloem); M]. Reference strain is SCYLP from Cuban sugarcane. DNA samples from this strain are available from the authors.

'*Candidatus* Phytoplasma caricae' (N.L. gen. n. *caricae* of *Carica*, the scientific generic name of papaya; epithet referring to the plant host) [(*Mollicutes*) NC; NA; O, wall-less; NAS (GenBank accession no. AY725234); oligonucleotide sequences of unique regions of 16S rRNA gene: 5′-AAA-3′, 5′-ATT-3′, 5′-AGGCGCC-3′ and 5′-GCGGATTTAGTC-ACTTTTCAGGC-3′; P (*Carica papaya*; phloem); M]. Reference strain is PAY from papaya. DNA samples from this strain are available from the authors.

## ACKNOWLEDGEMENTS

We thank the Royal Society for the provision of a short-term fellowship to Dr Yaima Arocha Rosete. We are grateful to Dr G. Firrao (University of Udine, Italy) for kindly providing DNA of Stolbur group phytoplasmas for comparison. We gratefully acknowledge Dr N. A. Harrison (University of Florida, USA) and Dr G. Firrao for their critical reading and revision of the manuscript. We are grateful to K. W. Coleman from Rothamsted Research for his technical assistance with restriction-map analysis. We deeply thank anonymous reviewers for their excellent helpful comments. This research was partly supported by the International Frontier for Science (IFS), Stockholm, Sweden, through a grant to Dr Y. Arocha Rosete. Work in the UK was done under Defra plant health licence no. PHL: 174B/4612(09/2003) and was supported by the Department for International Development, UK Global Plant Clinic.

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