Phylogenetic positions of phytoplasmas associated with dieback, yellow crinkle and mosaic diseases of papaya, and their proposed inclusion in 'Candidatus Phytoplasma australiense' and a new taxon, 'Candidatus Phytoplasma australasia'

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DNA extracted from three papaya (Carica papaya L.) plants, individually affected by dieback, yellow crinkle or mosaic diseases, was subjected to PCR using phytoplasma-specific primers to amplify the 16S rRNA gene plus 16S–23S rRNA intergenic spacer region. Near-complete DNA sequences obtained for the three PCR amplimers were subjected to phylogenetic analyses and direct sequence comparison with other phytoplasma 16S rDNA and 16S–23S spacer region DNA sequences. The papaya yellow crinkle (PpYC) and papaya mosaic (PpM) sequences were identical to each other, but distinctly different from the papaya dieback (PpDB) sequence, showing 90.3% identity in the 16S rDNA and 87.8% identity in the 16S–23S spacer region DNA sequences. A phylogenetic tree based on 16S rDNA sequences was calculated, in which PpYC and PpM are most closely related to the tomato big bud phytoplasma (TBB; 99.7% 16S rDNA sequence identity) from Australia, within subclade iii. This subclade consists of strains only reported occurring in the Southern Asian region and Australia, which indicates an Asian/Australasian origin. PpDB is most closely related to the Phormium yellow leaf phytoplasma from New Zealand (PYL; 99.9% identity) and the Australian grapevine yellows phytoplasma (AGY; 99.7% identity). These three phytoplasma strains form a distinct clade within subclade xii, which also includes the European strains STOL and VK as another distinct clade. The origin of the closely related but geographically separated AGY-like strains and STOL-like strains of subclade xii is unclear. It is proposed that phytoplasma strains PpDB, PYL and AGY be included in the previously described taxon 'Candidatus Phytoplasma australiense', and that PpYC, PpM and TBB be assigned to a new taxon, 'Candidatus Phytoplasma australasia'.

Keywords: phytoplasmas, phylogeny, papaya, 16S rDNA, classification

INTRODUCTION

Dieback, yellow crinkle and mosaic are three important diseases of papaya (Carica papaya L.) in Australia. Dieback, the most severe of these diseases, is responsible for annual plant losses of 10%, and up to 100% during epiphytotics, in central and Southern Queensland plantations (Glennie & Chapman, 1976). Yellow crinkle incidence is usually low and sporadic; however, epiphytotics can also occur. Papaya mosaic is generally of minor importance (Peterson et al., 1993; Simmonds, 1965). Incidences of dieback, yellow crinkle and possibly mosaic have recently been recorded in the Northern Territory (Conde et al., 1996). Transmission studies have demonstrated that the aetiological agent of yellow crinkle is the same as that causing tomato big bud in Australia (Greber, 1966). Viruses were first thought to be the pathogens causing

The EMBL accession numbers for the sequences reported in this paper are Y10095 (PpDB), Y10097 (PpYC) and Y10096 (PpM).
Phytoplasmas are cell-wall-less bacteria belonging to the class Mollicutes and are the proposed causative agents of diseases in several hundred plant species (McCoy et al., 1989). Phytoplasmas reside in the phloem tissue of the infected plant host and are transmitted by insect vectors, principally leafhoppers and planthoppers (Lee & Davis, 1992). Although phytoplasmas have been detected in affected plant tissues and insects with the use of technologies based on the transmission electron microscope, antibodies and nucleic acids (Lee & Davis, 1992), they are currently unable to be cultured in vitro. The association of phytoplasmas with dieback and mosaic in papaya has been based solely on PCR amplification of the 16S rRNA gene and adjacent regions using phytoplasma-specific primers (Davis et al., 1996; Gibb et al., 1996; Liu et al., 1996). The phytoplasma origin of PCR amplimers from dieback-, mosaic- and yellow-crinkle-affected papaya has been confirmed by restriction endonuclease and DNA sequence analyses (Gibb et al., 1996; White et al., 1997).

Due to the inability to culture phytoplasmas in vitro, taxonomic based on phenotypic characteristics has not been possible. To date, the most reliable classifications of phytoplasmas have been based on DNA restriction analysis and phylogenetic sequence analysis, usually of the PCR-amplified 16S rRNA gene plus 16S−23S rRNA intergenic spacer region (R. E. Davis et al., 1997; R. I. Davis et al., 1997; Gundersen et al., 1994, 1996; Lee et al., 1993; Namba et al., 1993; Schneider et al., 1993, 1995a, b; Seemüller et al., 1994). These analyses have allowed the provisional classification of phytoplasmas from Europe, North America, Asia and Australia. The phytoplasmas are phylogenetically distinct from the other members of the class Mollicutes, forming a monophyletic clade with the closest relatives belonging to the genus Acholeplasma (Gundersen et al., 1994; Kuske & Kirkpatrick, 1992; Namba et al., 1993; Seemüller et al., 1994). Among the phytoplasmas, Seemüller et al. (1994) identified five phylogenetic strain clusters, some of which could be divided into sub-groups. Schneider et al. (1995a) later distinguished a sixth strain cluster. In an alternative classification, Gundersen et al. (1994) identified 11 subclades within five strain clusters. Two further subclades were proposed by R. E. Davis et al. (1997) and Liefuing et al. (1996) within the classification of Gundersen et al. (1994). The classification systems of Seemüller et al. (1994) and Gundersen et al. (1994) are both commonly referred to, and can be directly compared with each other due to the inclusion of representative strains common to both systems.

The phytoplasmas associated with papaya dieback, yellow crinkle and mosaic have been grouped according to RFLP analysis of PCR-amplified 16S rDNA plus 16S−23S spacer region DNA (Gibb et al., 1996). In this paper, we report on the analysis of the DNA sequences of the 16S rRNA gene and 16S−23S spacer region of the phytoplasmas associated with Australian papaya dieback (PpDB), yellow crinkle (PpYC) and mosaic (PpM), and the positions of these strains in the current 16S rDNA phylogenetic classification systems.

A preliminary summary of this work has previously been presented at the Australasian Plant Pathology Society 11th Biennial Conference, Perth, Western Australia, 1997.

METHODS

Extraction of phytoplasma DNA. Papaya plants exhibiting characteristic symptoms of dieback, yellow crinkle or mosaic (Glennie & Chapman, 1976; Peterson et al., 1993; Simmonds, 1965) were collected from a commercial plantation at Yarwun, central Queensland, during January and February 1995. Nucleic acids were extracted from the midribs of fresh, symptomatic leaves as previously described by Liu et al. (1996). The dried DNA pellets were resuspended in 50 µl sterile, Millipore-filtered, distilled water and stored at −20°C. One extract was prepared from each of a dieback-, yellow-crinkle- and mosaic-affected papaya plant.

PCR amplification. Phytoplasma-specific PCR primers P1 and P7 (Table 1) were used to amplify a region approximately 1800 bp in length, consisting of the 16S rRNA gene, the 16S−23S rRNA intergenic spacer region and approximately 50 bp of the 5′ end of the 23S rRNA gene. Total PCR volumes were 100 µl and contained 200 µM of each dNTP, 0.4 µM of each primer, 1 x DNA polymerase reaction buffer, 1 U Tag DNA polymerase (Boehringer) and 5 µl template DNA solution. Each reaction mixture was covered with 50 µl sterile mineral oil (Sigma). Reactions were performed in a Minicycler (M. J. Research) with initial denaturation at 94°C for 2 min, followed by 40 cycles consisting of denaturation at 94°C for 1 min, annealing at 52°C for 30 s and extension at 72°C for 30 s, with extension in the final cycle for 2 min. Five microlitres of each PCR product was subjected to electrophoresis in a 1% (w/v) agarose gel stained with ethidium bromide and observed under UV illumination. A total of four PCR products was performed for each DNA extract and the amplification products were pooled for each disease.

DNA sequencing. The amplification products were purified for sequencing using the Promega Wizard PCR Preps spin column purification system, according to the manufacturer’s instructions for PCR product purification without a vacuum manifold. For each of the DNA extracts, the pooled PCR amplimers were eluted from the mini columns with 100 µl sterile, Millipore-filtered, distilled water.

Overlapping regions of both strands of the amplimers were sequenced using 13 primers typically used for sequencing bacterial 16S rRNA genes (Table 1) (Blackall et al., 1994; Bradford et al., 1996). The forward primer P3 (Table 1) was used in conjunction with P7 to sequence the 16S−23S rRNA spacer region. Direct cycle sequencing reactions were performed using the PRISM Ready Reaction DyeDeoxy Terminator Sequencing kit (Applied Biosystems). Three to
five microlitres of purified PCR product was used as template for the sequencing reactions. Reactions were performed in a Perkin Elmer model 480 thermal cycler and the thermal cycling profile was initial denaturation at 96 °C for 2 min, followed by 25 cycles consisting of denaturation at 96 °C for 30 s, reannealing at 50 °C for 15 s and extension at 60 °C for 4 min. Reaction products were purified by the chloroform method described in the manufacturer’s instructions for the sequencing kit and were electrophoresed and detected using an Applied Biosystems model 373A automated DNA sequencer.

Comparative sequence analysis. Initial sequence alignment and editing were done using the computer program SeqEd (Applied Biosystems). The overlapping sequence fragments were manually aligned against the Escherichia coli 16S rDNA sequences according to secondary structure (Lane, 1991), and were compiled to give the full 16S rDNA plus 16S–23S spacer region DNA sequence of the PCR amplimer from each of the three papaya disease DNA extracts.

Further analyses were conducted using programs available via the Australian National Genomic Information Service (ANGIS). The full 16S rDNA plus 16S–23S spacer region DNA sequences were subjected to BLAST (Altschul et al., 1990) analyses to search for similar sequences in the nucleic acid databases. All phytoplasma 16S rDNA sequences available in the nucleic acid databases, with a length of at least 1300 nucleotides, were used in the phylogenetic analyses (Table 2). Acholeplasma palmae, a closely related non-phytoplasma mollicute (R. E. Davis et al., 1997; Gundersen et al., 1994), was used as the outgroup. The reference sequences were aligned with the PpDB, PpYC and PpM sequences using CLUSTAL W (Thompson et al., 1994) and the ae2 editor (Larsen et al., 1993).

Phylogenetic trees were calculated using distance (DNADIST) and maximum parsimony (DNAPARS) method programs in PHYLIP version 3.5 (Felsenstein, 1993). Nucleotide positions at which a gap occurred in any of the aligned sequences were excluded from the analysis. An evolutionary distance matrix was calculated using the Jukes and Cantor-parameter model in DNADIST, and trees were constructed using the neighbouring method (NEIGHBOR). To quantify relative support for branches inferred from genetic distance analyses and parsimony, ‘bootstrap’ resampling (100 resamplings) was employed. A significance level of 95% was adopted for testing hypotheses proposed a priori (Felsenstein, 1985).

Phylogenetic distance trees were calculated from two data sets of 16S rDNA sequences. One set included the Japanese phytoplasma strains OY, TWB and RYD (Table 2), to give a total of 52 phytoplasma strains in the analyses and the comparison of 1251 nucleotide positions. A second data set excluded the Japanese phytoplasmas, allowing the analysis of only 49 phytoplasma strains, but enabling the comparison of 1353 nucleotide positions. The phylogenetic tree generated from the second data set is presented in the results since it is based on more sequence information than the tree based on the first data set. Using the ae2 editor, a similarity matrix was constructed by direct pairwise comparison of all phytoplasma 16S rDNA sequences used for phylogenetic inferences. The PpDB, PpYC and PpM 16S–23S spacer region DNA sequences were aligned and compared with 22 other available phytoplasma spacer region sequences (Table 2) using ae2. A similarity matrix was constructed as for the 16S rDNA sequences.

**RESULTS**

**DNA sequences**

Almost the entire P1/P7 PCR amplimer was sequenced for each of the PpDB (1761 bp), PpYC (1799 bp) and PpM (1797 bp) phytoplasmas. Near-complete 16S rDNA sequences were obtained, except for up to 12 bp at the 5' end of the 16S rRNA gene of all three amplimers. The 16S–23S spacer region DNA sequences were 208 bp in length for PpDB and 222 bp in length for PpYC and PpM. Thirty-two base pairs of the 5' end of the 23S rRNA gene was determined for PpDB and 56 bp was determined for PpYC and PpM.

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**Table 1. PCR amplification and sequencing primers**

<table>
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<th>Primer</th>
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<tbody>
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<td>AAAGATTGTGATCCGGCTAG</td>
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<tr>
<td>27f</td>
<td>GAGGGTGTGATCCGGCTAG</td>
<td>Dorsch &amp; Stackebrandt (1992)</td>
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<td>342r</td>
<td>CTGCTGCTGCGCCCTAG</td>
<td>Lane (1991)</td>
</tr>
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<td>357f</td>
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<tr>
<td>803f</td>
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<td>803f</td>
<td>ATTAGAGACCCGGTGTAG</td>
<td>Stackebrandt &amp; Charfreitag (1990)</td>
</tr>
<tr>
<td>907r</td>
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<td>1492r</td>
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</tr>
<tr>
<td>P3</td>
<td>GGATGATACATCTCCTT</td>
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<tr>
<td>P7</td>
<td>GTGCTCCTTCATCGGCTTT</td>
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</tr>
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Table 2. Associated diseases and accession numbers of 16S rDNA and 16S–23S spacer region DNA sequences of phytoplasma strains used in this study

<table>
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<th>Strain*</th>
<th>Associated plant disease and origin</th>
<th>Sequence†</th>
<th>Accession no.</th>
<th>Reference</th>
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<td>16S, SR</td>
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<td>X95706</td>
<td>Padovan et al. (1996)</td>
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<td>X83432</td>
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Table 2. (cont.)

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<th>Accession no.</th>
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<td>CP</td>
<td>Clover proliferation; Alberta, Canada</td>
<td>16S</td>
<td>L33761</td>
<td>Gundersen et al. (1994)</td>
</tr>
<tr>
<td>BLL</td>
<td>Eggplant (brinjal) little leaf; India</td>
<td>16S, SR</td>
<td>X83431</td>
<td>Schneider et al. (1995a)</td>
</tr>
<tr>
<td>BLTVA</td>
<td>Beet-leafhopper-transmitted virescence agent; CA, USA</td>
<td>SR</td>
<td>U54987</td>
<td>Smart et al. (1996)</td>
</tr>
<tr>
<td>EY</td>
<td>Elm yellows; New York, USA</td>
<td>16S</td>
<td>L33763</td>
<td>Gundersen et al. (1994)</td>
</tr>
<tr>
<td>ULW</td>
<td>Elm yellows; France</td>
<td>16S</td>
<td>X68376</td>
<td>Schneider et al. (1993)</td>
</tr>
<tr>
<td>ULW</td>
<td>Elm yellows; France</td>
<td>SR</td>
<td>U54991</td>
<td>Smart et al. (1996)</td>
</tr>
<tr>
<td>FD</td>
<td>Flavescence dorée of grapevine; France</td>
<td>16S, SR</td>
<td>X76560</td>
<td>Seemüller et al. (1994)</td>
</tr>
<tr>
<td>LWB</td>
<td>Loofah witches' broom; Taiwan</td>
<td>16S</td>
<td>L33764</td>
<td>Gundersen et al. (1994)</td>
</tr>
<tr>
<td>A. palmae</td>
<td></td>
<td>16S</td>
<td>L33734</td>
<td>Gundersen et al. (1994)</td>
</tr>
</tbody>
</table>

* Strains are presented in vertical order as they appear in Fig. 1, or for strains listed with only SR sequences, presented under phylogenetically similar strains.
† 16S, 16s rRNA gene; SR, 16S–23S rRNA spacer region.
‡ Database record was the only available reference at the time of analysis.
§ Geographical origin could not be determined from database record.

The PpYC and PpM sequences were identical to each other.

Phylogenetic analysis of 16S rDNA sequences

A phylogenetic distance tree was calculated from a data set which included the strains OY, TWB and RYD (Fig. 1). This tree and that calculated from a data set which included strains OY, TWB and RYD both exhibited branching orders similar to previously published trees (Gundersen et al., 1994; Liefting et al., 1996; Marcone et al., 1996; Schneider et al., 1995a). High bootstrap values (Fig. 1) supported the same major phylogenetic clusters identified by Seemüller et al. (1994) and Schneider et al. (1995a), and the phylogenetic subclades identified by Gundersen et al. (1994) and R. E. Davis et al. (1997).

In this phylogenetic analysis (Fig. 1), PpDB was most closely related to the Australian grapevine yellows strain AGY and the New Zealand Phormium yellow leaf strain PYL phytoplasmas within subclade xii (R. E. Davis et al., 1997), which corresponds to the stolbur sub-group of the aster yellows strain cluster, cluster I (Seemüller et al., 1994). Bootstrap values of 100% (Fig. 1) support the PpDB/AGY/PYL clade as being distinct from the STOL/VK clade. Direct pairwise comparison of the 16S rDNA sequences showed that the PpDB sequence was most similar to those of PYL (99.9%), AGY (99.7%), STOL (98.3%) from Serbia and VK (98.3%) from Germany. Similarity of the PpDB 16S rDNA sequence with those of the aster yellows strains ranged from 95.9% (AAY) to 97.3% (KV).

The PpYC and PpM phytoplasma 16S rDNA sequences were identical to each other. In the phylogenetic analysis (Fig. 1), PpYC was most closely related to tomato big bud strain TBB from Australia, within the peanut witches’ broom subclade (subclade iii) described by Gundersen et al. (1994), which corresponds to the faba bean phylloidy strain cluster, cluster VI (Schneider et al., 1995a). PpYC, together with TBB, peanut witches’ broom strain PnWB from Taiwan, sweet potato witches’ broom strain SPWB from Taiwan, sunn hemp witches’ broom strain SUNHP from Thailand and sweet potato little-leaf strain SPLL from Australia, form a clade (distance bootstrap value 97%) distinct from lime witches’ broom disease strain WBDL from Oman and fava bean phylloidy strain FBP from Sudan (bootstrap value 100%) (Fig. 1). Direct pairwise comparisons of sequences showed that the PpYC 16S rDNA sequence was most similar to those of TBB (99.7%), PnWB (99.7%), SUNHP (99.4%), SPWB (99.1%) and SPLL (99.1%). The PpYC sequence was 98.8% similar to the WBDL sequence and 98.6% similar to the FBP sequence, while only 92.3% similar to the sequence of strain WX in subclade iv. Direct sequence comparison also showed that the PpYC 16S rDNA sequence is 90.3% similar to the PpDB sequence.

16S rRNA signature sequences

R. E. Davis et al. (1997) described three 16S rRNA signature sequences that are unique to subclade xii phytoplasma strains and two signature sequences that were unique to the Australian grapevine yellows strain AUSGY. Although the available AUSGY 16S rRNA sequence is shorter than that of Australian grapevine yellows strain AGY (Padovan et al., 1996), the two
Fig. 1. Phylogenetic distance tree of PpDB, PpYC and other phytoplasmas based on the comparative analysis of 1353 nucleotide positions of 16S rRNA gene sequences, with two phylogenetic group classification systems presented [subclades of Gundersen et al. (1994), R. E. Davis et al. (1997) and this paper, and strain clusters of Seemüller et al. (1994) and Schneider et al. (1995a)]. Bar, phylogenetic distance of 10%. Names of state or country of origin are in parentheses after strain name abbreviations (as presented in Table 2). Bootstrap values greater than 50% (100 bootstrap resamplings) from distance (upper) and parsimony (lower) analyses are presented at the nodes. Acholeplasma palmae was used as the outgroup in the analyses.
sequences are identical within the matching regions. Since the AGY sequence is longer, it was used in the current phylogenetic analysis. The PpDB and PYL phytoplasma 16S rRNA sequences (this study; Liefting et al., 1996) have the same signature sequences described for AUSGY, thus distinguishing PpDB, PYL and AGY from STOL and VK, within subclade xii. Gundersen et al. (1994) reported two 16S rRNA signature sequences that are unique to subclade iii. These signature sequences occur only in PpYC, PpM and the other strains in subclade iii (Fig. 1). Additionally, the following two unique sequences that distinguish PpYC, PpM and TBB from other phytoplasma strains were found: 5'-TAAAAGGCAATCTTTATTAC-3' at positions 178-195 (numbering corresponding to 16S rRNA gene sequence of OAY; Lim & Sears, 1989) and 5'-CAAGGAAGAAAGCAGATGCGGAAAATGGCCAGACCTATTGTTTTTTT-3' at positions 444-477. PnWB, SPWB and SUNHP differ from the first unique sequence by having the same single nucleotide substitution, 5'-TAAAAGGCAATCTTTATCATC-3', and SPLL differs by an additional nucleotide substitution, 5'-TAGAAGGCGATCTTGTATC-3'. SPLL contains the second unique oligonucleotide sequence exactly, whereas PnWB, SPWB and SUNHP differ by the same single nucleotide substitution, 5'-CGAGG(25 nucleotides, see above)GTTT-3'.

**Analysis of 16S–23S spacer region DNA sequences**

All 25 phytoplasma sequences that were compared had tRNA\textsuperscript{ile} (GAT anticodon) genes 77 bp in length. The tRNA\textsuperscript{ile} sequences of PpDB, PpYC and PpM were identical to that of the OAY phytoplasma (Lim & Sears, 1989). The similarity of sequences external to the tRNA\textsuperscript{ile} gene in different phytoplasma strains reflected the same grouping observed in the phylogenetic tree based on the 16S rDNA sequence (data not shown). Direct pairwise comparisons of whole spacer region sequences showed PpDB to be most similar to PYL (100%) and AGY (99.6%), while showing only about 95% identity with subclade iv strains SAY, RPh and OAY. The 16S–23S spacer region DNA sequences of the PpYC and PpM phytoplasmas were identical to each other. PpYC and PpM are most similar to TBB (99.6%) and SPLL (99.6%), while showing 98.9% and 98.4% identity with WBDL and FBP, respectively, and only 83.0% identity with the subclade iv strain WX. The PpYC spacer region sequence is 87.8% similar to the PpDB sequence.

**DISCUSSION**

**Phytoplasma phylogenetic classification**

The phylogenetic tree presented in this paper (Fig. 1) is based on all near-complete phytoplasma 16S rDNA sequences that were available on public nucleic acid databases at the time of analysis. This represents an advance on earlier phylogenetic classifications, since previous studies (R. E. Davis et al., 1997; Liefting et al., 1996; Marcone et al., 1996; Padovan et al., 1996; Schneider et al., 1995a; Zreik et al., 1995) included only representative strains from the subclades and strain clusters defined by Gundersen et al. (1994) and Seemüller et al. (1994). By including all available phytoplasma 16S rDNA sequences in a single phylogenetic distance tree, the relationships of all strains to each other are clear, and the two current classification schemes can be directly compared (Fig. 1). Although the original phylogenetic trees published by Gundersen et al. (1994) and Seemüller et al. (1994) included only some strains from each of the distinct clades, the clustering of strains is sufficient to identify corresponding clades between the two systems.

Based on near-complete 16S rDNA sequences of 21 phytoplasma strains, Seemüller et al. (1994) originally identified the following five primary clusters: I, the aster yellows strain cluster; II, the apple proliferation strain cluster; III, the Western X-disease strain cluster; IV, the sugar cane white leaf strain cluster; and V, the elm yellows strain cluster. Schneider et al. (1995a) later distinguished the faba bean phyllody strain cluster, cluster VI, which includes strain SUNHP, previously included in cluster III (Seemüller et al., 1994). Based on near-complete 16S rDNA sequences of 19 phytoplasma strains, Gundersen et al. (1994) also recognized five major phylogenetic groups; however, two of these groups were different to those identified by Seemüller et al. (1994) due to the analysis of different phytoplasma strains. The five main groups distinguished by Gundersen et al. (1994) were further refined into the following 11 subclades by analysis of partial 16S rDNA sequences of 30 phytoplasma strains: i, aster yellows strains; ii, apple proliferation strains; iii, peanut witches' broom strains; iv, X-disease strains; v, strain RYD; vi, strain PPWB; vii, strain LY; viii, strain ASHY; ix, clover proliferation strains; x, elm yellows strains; and xi, strain LiWB. R. E. Davis et al. (1997) later added strains STOL, VK and AUSGY, which formed a distinct subclade, designated subclade xii, most closely related to subclade i, the aster-yellows-like strains.

Liefting et al. (1996) included strains PYL, STOL and VK in a phylogenetic analysis and recognized that these three strains formed a subclade, which they named subclade xii. However, R. E. Davis et al. (1997) defined this subclade as subclade xii, and this classification has been adopted in the current paper. Liefting et al. (1996) also included strains LY, LDY and LD. Although LD clustered with LY and LDY, it was separated from them by deep branches, such that LD could be assigned to its own subclade. Liefting et al. (1996) proposed the name subclade xii; however, 'subclade xii' is more appropriate as the former designation conflicts with the naming of a different subclade by R. E. Davis et al. (1997) (Fig. 1). Also, Liefting et al. (1996) found that LY, LDY, LD and PPWB clustered together, and proposed naming this group as a seventh strain cluster in the classification system of Seemüller et al. (1994) and Schneider et al. (1995a). We agree that these strains, along with
CPPWB, do cluster together (Fig. 1); however, non-
significant bootstrap values (less than 95%) do not
support a distinct strain cluster. At present it would be
to best recognize that these strains form three sub-
clades, vi (PPWB and CPPWB), vii (LY and LDY) and
xiii (LD), but should not be recognized as a distinct
clade within the classification system of Seemuller et al.
(1994) and Schneider et al. (1995a).

PpDB and related strains

PpDB is clearly related to the AGY and PYL phyto-
plasmas within subclade xii. Previously, restriction
endonuclease analysis of the P1/P7 amplicer from
PpDB nucleic acid extracts revealed the similarities
with AGY, STOLF (stolbur of tomato from France)
and AAY (R. I. Davis et al., 1997; Gibb et al., 1996).
Previous sequence analysis of a 500 bp region of the
16S rRNA gene and the 16S-23S spacer region also
revealed that the PpDB phytoplasma was closely
related to STOL, VK and SAY (White et al., 1997).
Restriction endonuclease analysis of the PCR-ampli-
fied tuf gene further supported the close genetic
relationship between the PpDB and AGY phyto-
plasmas (Padovan et al., 1996). These studies also
indicated that the PpDB/AGY strains are distinct
from the STOL/VK strains. The results of 16S rDNA
and 16S–23S spacer region DNA sequence analyses
presented in this paper confirm the close genetic
relatedness of PpDB to AGY, and clearly demonstrate
the close relationship of these phytoplasma strains to
the PYL phytoplasma from New Zealand. Within
subclade xii, PpDB, AGY and PYL form a clade
distinguished from the European strains STOL and VK
(Fig. 1). Based on the 16S rDNA sequence data, PpDB
and PYL can also be included in the 16S rDNA RFLP
subgroup 16SrI-J, with AUSGY and AGY (R. E. Davis
et al., 1997).

PpYC, PpM and related strains

Previous restriction endonuclease analysis (Gibb et al.,
1996) and sequence analysis (White et al., 1997) of
amplified 16S rDNA and 16S–23S spacer region DNA
revealed identity between those regions of DNA of the
PpYC and PpM phytoplasmas. Gibb et al. (1996) and
White et al. (1997) speculated that the same strain, or
very similar strains of phytoplasmas, were responsible
for papaya yellow crinkle and mosaic, and that the
differences in disease symptoms may be due to dif-
ferences in plant physiological and or pathological
factors. Investigation of other genomic sequences may
reveal differences between PpYC and PpM. The PpYC
and PpM phytoplasmas were previously found to be
similar to TBB and SPLL from Australia, and
SUNHP, SEPT (sesame phyllody), CLP (Cleome
viscosa phyllody) and CROP (crotalaria phyllody)
from Thailand, by RFLP analysis of P1/P7 amplimers
(R. I. Davis et al., 1997; Gibb et al., 1996), and similar
to PnWB, SUNHP and WBDL by sequence analysis
(White et al., 1997). Greber (1966) had previously
demonstrated the close relationship between the
papaya yellow crinkle and tomato big bud agents by
dodder transmission experiments.

The results presented in this paper confirm that the
PpYC and PpM phytoplasmas are most closely related
to the Australian TBB and SPLL phytoplasmas, as
well as the South-East Asian SUNHP, PnWB and
SPWB phytoplasmas, which belong to subclade iii
(Gundersen et al., 1994) or strain cluster VI (Schneider
et al., 1995a). Within this clade are the FBP and
WBDL phytoplasmas from Sudan and Oman, re-
spectively. Previously, strains FBP and WBDL were
each grouped only with SUNHP in separate pub-
lications (Schneider et al., 1995a; Zreik et al., 1995).
The phylogenetic tree presented in Fig. 1, along with
pairwise comparisons of the 16S and 16S–23S spacer
region sequences (99.5% identity in 16S rDNA se-
quence and 99.6% identity in the 16S–23S spacer
region sequence), clearly show that strains FBP and
WBDL are more closely related to each other than to
the other subclade iii strains.

Origins of Australian phytoplasma strains

In a survey of Australian phytoplasma diseases, R. I.
Davis et al. (1997) found that the majority of diseases
occurring in the 38 plant species tested were associated
with the TBB strain. The closely related SPLL strain
occurred only in sweet potato (Ipomoea batatas) and
pigeon pea (Cajanus cajan). The only other strain
detected was the AGY/PpDB strain. Due to the
ubiquity of the TBB strain and the close relationship of
TBB and SPLL strains to other phytoplasma strains
(subclade iii strains) occurring only in Southern Asia,
R. I. Davis et al. (1997) hypothesized an Australasian
origin of TBB and SPLL strains. WBDL and the
strains represented by FBP (Schneider et al., 1995a)
form a distinct clade within subclade iii, and have a
recorded geographical distribution extending from
Thailand in South-East Asia to Sudan in North-East
Africa. Based on this distinct geographical distribu-
tion, it seems very likely that the subclade iii
phytoplasmas originated and evolved in Southern
Asia, with an apparent evolutionary and geographical
divergence to form the South-West Asian strains (FBP
and WBDL) and the South-East Asian/Australasian
strains.

The AGY-like strains have only been detected in
grapevines (Vitis vinifera) and papaya in Australia and
New Zealand flax (Phormium tenax). The next most
closely related strains, the STOL-like strains, have
only been found in Europe. Currently, there is no
explanation for the apparent close relatedness yet
distinct geographical separation of the AGY- and
STOL-like strains. Although, at present, it seems that
the AGY-like strains are endemic to Australia and
New Zealand, their actual origin and evolutionary
relationship with the STOL-like strains can only be
speculated until they are detected in more plant host
and/or insect vector species.
Phytoplasma taxa

Since the proposed to use the name 'phytoplasma' for the plant-pathogenic mycoplasma-like organisms (Tully, 1993; Sears & Kirkpatrick, 1994), there has been increasing support for recognizing the phytoplasmas as a distinct genus (R. E. Davis et al., 1997; Gundersen et al., 1994; Zreik et al., 1995). Also, Gundersen et al. (1994) proposed that each phylogenetically distinct subclade should represent at least distinct species.

R. E. Davis et al. (1997) defined the provisional taxon 'Candidatus Phytoplasma australiense' based on 16S rRNA signature sequences of AUSSY, which have been found in strains PpDB, AGY and PYL, thus supporting a distinct group of closely related strains. Due to their close genetic relationship and distinct geographical range, we propose that strains PpDB, AGY and PYL be provisionally included in the taxon 'Candidatus Phytoplasma australiense'.

Zreik et al. (1995) proposed the taxon 'Candidatus Phytoplasma aurantifolia' based on the WBDL strain 16S rDNA sequence. Phylogenetic analysis and direct pairwise sequence comparison in the present study have shown that strain FBP and strain WBDL are more closely related to each other than to any other characterized strains. The 16S rRNA gene oligonucleotide sequence listed by Zreik et al. (1995) to define strain WBDL as 'Candidatus Phytoplasma aurantifolia' differs from the corresponding sequence of other subclade iii phytoplasma strains by two nucleotide substitutions and differs from that of FBP by a single nucleotide substitution. Despite this single nucleotide difference in the definitive oligonucleotide, the current phylogenetic study statistically supports (100% bootstrap; Fig. 1) the inclusion of FBP and WBDL in a taxon distinct from the other subclade iii strains. Thus we suggest that strain FBP is sufficiently similar to WBDL to provisionally be included in the taxon 'Candidatus Phytoplasma aurantifolia'.

Based on the guidelines of Murray & Schleifer (1994), we propose that the PpYC, PpM and TBB phytoplasmas be assigned to a new Candidatus species with the following description: 'Candidatus Phytoplasma australasia' [(Mollicutes) NC; NA; O; NAS (EMBL Y10097), oligonucleotide sequences of unique regions of the 16S rRNA gene 5'-TAAAGGCACTTTTATC-3' and 5'-CAAGGAAGAAAGCAATGGCGGAACCATTGGTTT-3'; P (Carica papaya, Lycopersicon esculentum, phloem); M;] Although strains PnWB, SPWB, SUNHP and SPLL from subclade iii have minor variations in sequence regions that are unique to strains in 'Candidatus Phytoplasma australasia' (PpYC, PpM and TBB), we suggest that they be provisionally included in this taxon because of the close phylogenetic relationships of all these strains (Fig. 1) and their distinct geographic range from South-East Asia to Australia.

As an economically important group of mollicutes, we support efforts to 'facilitate reference to (each) unique phytoplasma lineage' (R. E. Davis et al., 1997) by describing putative taxa, despite the inability to culture these organisms. Future taxonomic definitions which are based primarily on nucleic acid sequence information should be based on more than one conserved gene. Sequence analysis of conserved phytoplasma genes, in addition to the 16S rRNA gene, is likely to reveal more clearly the relationships between those strains which we have suggested be provisionally placed in the three discussed Candidatus species. Geographic and host range should also be considered as important criteria. Fortunately, due to their obligate parasitic nature, it is likely that phytoplasma genetic diversity will reflect the biogeography of their hosts. Finally, for practical reasons, the taxonomic system may be weighted with taxa for which there is a need to refer to as distinct pathogens of cultivated plants. For example, two geographically isolated strains of economic importance, with greater than 99% sequence similarity or significant phylogenetic confidence (bootstrap) values, may be distinguished as separate species, while two co-located strains or non-economic strains may remain grouped within a single species.

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D. T. White and others


