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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 (Condé *et al.*, 1996). Transmission studies have demonstrated that the aetiologic 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).

tomato big bud and papaya yellow crinkle; however, it was later shown by transmission electron microscopy that phytoplasmas are associated with both diseases (Bowyer *et al*., 1969; Gowanlock *et al*., 1976). Based on results of PCR detection, phytoplasmas have also been implicated as the aetiologic agents of papaya dieback and papaya mosaic (Davis *et al*., 1996; Gibb *et al*., 1996; Liu *et al*., 1996).

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 phytoplasmal 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*, taxonomy 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 Liefting *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 × DNA polymerase reaction buffer, 1 U *Taq* 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 1·0% (w/v) agarose gel, stained with ethidium bromide and observed under UV illumination. A total of four PCRs 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

Table 1. PCR amplification and sequencing primers

Primer	Nucleotide sequence* (5′ → 3′)	Reference
P1	AAGAGTTTGATCCTGGCTCAGGATT	Deng & Hiruki (1991)
27f	GAGTTTGATCCTGGCTCAG	Dorsch & Stackebrandt (1992)
342r	CTGCTGCSYCCCGTAG	Lane (1991)
357f	CTCCTACGGGAGGCAGCAG	Lane (1991)
519r	GWATTACCGCGGCKGCTG	Lane (1991)
530f	GTGCCAGCMGCCGCGG	Lane (1991)
787r	CTACCAGGGTATCTAAT	Stackebrandt & Charfreitag (1990)
803f	ATTAGATACCCTGGTAG	Stackebrandt & Charfreitag (1990)
907r	CCGTCAATTCMTTTRAGTTT	Lane (1991)
926f	AAACTYAAAKGAATTGACGG	Lane (1991)
1100r	GGGTTGCGCTCGTTG	Lane (1991)
1114f	GCAACGAGCGCAACCC	Lane (1991)
1392r	ACGGGCGGTGTGTRC	Lane (1991)
1492r	TACGGYTACCTTGTTACGACTT	Lane (1991)
P3	GGATGGATCACCTCCTT	Schneider *et al.* (1995b)
P7	CGTCCTTCATCGGCTCTT	Schneider *et al.* (1995b)

* M = C:A, Y = C:T, K = G:T, R = A:G, S = G:C, W = A:T; all 1:1.

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 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 neighbour-joining 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.

Table 2. Associated diseases and accession numbers of 16S rDNA and 16S–23S spacer region DNA sequences of phytoplasma strains used in this study

Strain*	Associated plant disease and origin	Sequence†	Accession no.	Reference
PpDB	Papaya dieback; Queensland, Australia	16S, SR	Y10095	This paper
AGY	Grapevine yellows; South Australia, Australia	16S, SR	X95706	Padovan *et al.* (1996)
PYL	*Phormium* yellow leaf (*rrnB* operon); New Zealand	16S	U43570	Liefting *et al.* (1996)
PYL	*Phormium* yellow leaf; New Zealand	SR	U43571	Liefting *et al.* (1996)
STOL	Stolbur of pepper; Serbia	16S	X76427	Seemüller *et al.* (1994)
VK	Vergilbungskrankheit (grapevine yellows); Germany	16S	X76428	Seemüller *et al.* (1994)
AAY	American aster yellows; FL, USA	16S	X68373	Schneider *et al.* (1993)
SAY	Severe Western aster yellows; CA, USA	16S, SR	M86340	Kuske & Kirkpatrick (1992)
AY1	Maryland aster yellows; MD, USA	16S	L33767	Gundersen *et al.* (1994)
OY	Onion yellows; Japan	16S	D12569	Namba *et al.* (1993)
RpPh	Winter oilseed rape phyllody; Czech Republic	16S, SR	U89378	–‡
OAY	*Oenothera* (Michigan) aster yellows; MI, USA	16S	M30790	Lim & Sears (1989)
OAY	*Oenothera* (Michigan) aster yellows; MI, USA	SR	–	Lim & Sears (1989)
BB	Tomato big bud; AR, USA	16S	L33760	Gundersen *et al.* (1994)
CCPh	Clover phyllody; Ontario, Canada	16S	L33762	Gundersen *et al.* (1994)
KV	Clover phyllody; Germany	16S	X83870	–‡
ACLR	Apricot chlorotic leaf roll; Spain	16S	X68338	Schneider *et al.* (1993)
PPER	European stone fruit yellows of peach; Germany	16S	X68374	Schneider *et al.* (1993)
ESF-PCH	European stone fruit yellows of peach; Germany	SR	U54988	Smart *et al.* (1996)
ESFY	European stone fruit yellows of apricot; Czech Republic	16S, SR	Y11933	–‡
AT	Apple proliferation; Germany	16S	X68375	Schneider *et al.* (1993)
AT	Apple proliferation; Germany	SR	U54985	Smart *et al.* (1996)
PD	Pear decline; Germany	16S	X76425	Seemüller *et al.* (1994)
PD-308	Pear decline; Germany	SR	U54989	Smart *et al.* (1996)
PYLR2	Peach yellow leaf roll; CA, USA	SR	U54990	Smart *et al.* (1996)
APS	Apple proliferation; Spain	16S	X76426	Seemüller *et al.* (1994)
SPAR	Spartium witches' broom; Italy	16S, SR	X92869	Marcone *et al.* (1996)
BAWB	Black alder witches' broom; Germany	16S	X76431	Seemüller *et al.* (1994)
PpYC	Papaya yellow crinkle; Queensland, Australia	16S, SR	Y10097	This paper
PpM	Papaya mosaic; Queensland, Australia	16S, SR	Y10096	This paper
TBB	Tomato big bud; South Australia, Australia	16S, SR	Y08173	Gibb *et al.* (1998)
PnWB	Peanut witches' broom; Taiwan	16S	L33765	Gundersen *et al.* (1994)
SPWB	Sweet potato witches' broom; Taiwan	16S	L33770	Gundersen *et al.* (1994)
SUNHP	Sunn hemp witches' broom; Thailand	16S	X76433	Seemüller *et al.* (1994)
SPLL	Sweet potato little leaf; Northern Territory, Australia	16S, SR	X90591	Padovan *et al.* (1996)
WBDL	Witches' broom disease of lime; Oman	16S, SR	U15442	Zreik *et al.* (1995)
FBP	Faba bean phyllody; Sudan	16S, SR	X83432	Schneider *et al.* (1995a)
WX	Western X-disease; CA, USA	16S	L04682	Schneider *et al.* (1993)
WX	Western X-disease; CA, USA	SR	U54992	Smart *et al.* (1996)
CX	Canadian peach X-disease; Ontario, Canada	16S	L33733	Gundersen *et al.* (1994)
VAC	Vaccinium witches' broom; Germany	16S	X76430	Seemüller *et al.* (1994)
TWB	Tsuwabuki witches' broom; Japan	16S	D12580	Namba *et al.* (1993)
CYE	Clover yellow edge; Ontario, Canada	16S	L33766	Gundersen *et al.* (1994)
ICPh	Clover phyllody; Italy	16S	X77482	Firrao *et al.* (1996)
SCWL	Sugar cane white leaf; Thailand	16S	X76432	Seemüller *et al.* (1994)
RYD	Rice yellow dwarf; Japan	16S	D12581	Namba *et al.* (1993)
BVK	Blütenverkleinerung (from leafhopper *Psammotettix cephalotes*); Germany	16S	X76429	Seemüller *et al.* (1994)
CIRP	From *Cirsium arvense*§	16S, SR	X83438	–‡
PPWB	Pigeon pea witches' broom; FL, USA	16S	L33735	Gundersen *et al.* (1994)
CPPWB	Caribbean pigeon pea witches' broom§	16S	U18763	–‡
LY	Coconut lethal yellowing; FL, USA	16S	U18747	–‡
LDY	Yucatan coconut lethal decline; Mexico	16S	U18753	–‡
LD	Coconut lethal disease; Tanzania	16S	X80117	–‡

Table 2. (*cont.*)

Strain*	Associated plant disease and origin	Sequence†	Accession no.	Reference
ASHY	Ash yellows; New York, USA	16S	X68339	Schneider *et al.* (1993)
ASHY	Ash yellows; New York, USA	SR	U54986	Smart *et al.* (1996)
CP	Clover proliferation; Alberta, Canada	16S	L33761	Gundersen *et al.* (1994)
BLL	Eggplant (brinjal) little leaf; India	16S, SR	X83431	Schneider *et al.* (1995a)
BLTVA	Beet-leafhopper-transmitted virescence agent; CA, USA	SR	U54987	Smart *et al.* (1996)
EY	Elm yellows; New York, USA	16S	L33763	Gundersen *et al.* (1994)
ULW	Elm yellows; France	16S	X68376	Schneider *et al.* (1993)
ULW	Elm yellows; France	SR	U54991	Smart *et al.* (1996)
FD	Flavescence dorée of grapevine; France	16S, SR	X76560	Seemüller *et al.* (1994)
LfWB	Loofah witches' broom; Taiwan	16S	L33764	Gundersen *et al.* (1994)
A. palmae		16S	L33734	Gundersen *et al.* (1994)

*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 excluded 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 phyllody 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 faba bean phyllody 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

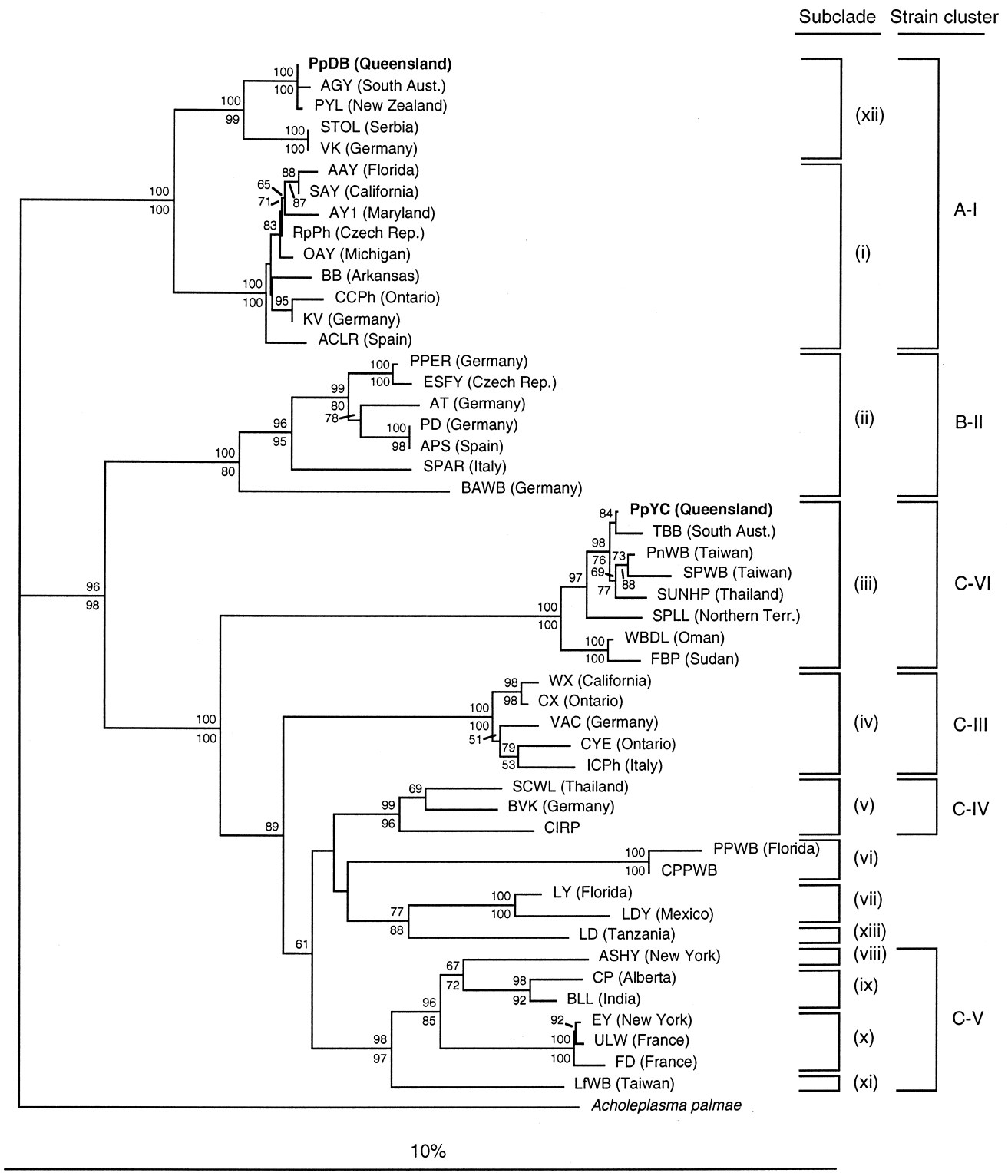

Fig. 1. Phylogenetic distance tree of PpDB, PpYC and other phytoplasmas based on the comparative analysis of 1353 nucleotide positions of 16S rRNA gene sequences, with two phylogenetic group classification systems presented [subclades of Gundersen *et al.* (1994), R. E. Davis *et al.* (1997) and this 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′-TAAAAGGCATCT-TTTATC-3′ at positions 178–195 (numbering corresponding to 16S rRNA gene sequence of OAY; Lim & Sears, 1989) and 5′-CAAGGAAGAAAAGCAA-ATGGCGAACCATTTGTTT-3′ at positions 444–477. PnWB, SPWB and SUNHP differ from the first unique sequence by having the same single nucleotide substitution, 5′-TAAAAGGCATCTT**G**T-ATC-3′, and SPLL differs by an additional nucleotide substitution, 5′-TA**G**AAGGCATCTT**G**TATC-3′. SPLL contains the second unique oligonucleotide sequence exactly, whereas PnWB, SPWB and SUNHP differ by the same single nucleotide substitution, 5′-C**G**AGG(25 nucleotides, see above)GTTT-3′.

Analysis of 16S–23S spacer region DNA sequences

All 25 phytoplasma sequences that were compared had $tRNA^{Ile}$ (GAT anticodon) genes 77 bp in length. The $tRNA^{Ile}$ sequences of PpDB, PpYC and PpM were identical to that of the OAY phytoplasma (Lim & Sears, 1989). The similarity of sequences external to the $tRNA^{Ile}$ 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 i strains SAY, RpPh 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 LfWB. 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 xiii. 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 xiii' 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 best to recognize that these strains form three subclades, 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 Seemüller *et al.* (1994) and Schneider *et al.* (1995a).

PpDB and related strains

PpDB is clearly related to the AGY and PYL phytoplasmas within subclade xii. Previously, restriction endonuclease analysis of the P1/P7 amplimer 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-amplified *tuf* gene further supported the close genetic relationship between the PpDB and AGY phytoplasmas (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 distinct 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 differences 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, respectively. Previously, strains FBP and WBDL were each grouped only with SUNHP in separate publications (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 sequence 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 distribution, 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 proposal 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 AUSGY, which have since 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′-TAAAAGGCATCTTTTATC-3′ and 5′-CAAGGAAGAAAAGCAAATGGCGAACCATTTGTTT-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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