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Short communication

'*Candidatus* Phytoplasma australiense' is the phytoplasma associated with Australian grapevine yellows, papaya dieback and *Phormium* yellow leaf diseases

Lia W. Liefting[1], Anna C. Padovan[2], Karen S. Gibb[2], Ross E. Beever[3], Mark T. Andersen[1], Richard D. Newcomb[1], David L. Beck[1] and Richard L.S. Forster[1]

[1*] *The Horticulture and Food Research Institute of NZ Ltd., Mt Albert Research Centre, Private Bag 92169, Auckland, New Zealand (Fax: +649 815 4201);* [2] *Faculty of Science, Northern Territory University, Darwin 0909, Australia;* [3] *Manaaki Whenua-Landcare Research, Mt Albert Research Centre, Private Bag 92170, Auckland, New Zealand;* * *Author for correspondence*

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Abstract

Sequence comparisons and phylogenetic analysis of the 16S rRNA genes and the 16S/23S spacer regions of the phytoplasmas associated with Australian grapevine yellows, papaya dieback and *Phormium* yellow leaf diseases revealed minimal nucleotide differences between them resulting in the formation of a monophyletic group. Therefore, along with Australian grapevine yellows, the phytoplasmas associated with *Phormium* yellow leaf and papaya dieback should also be considered as '*Candidatus* Phytoplasma australiense'.

Abbreviations: AGY or AUSGY – Australian grapevine yellows phytoplasma; PAUP – phylogenetic analysis using parsimony; PDB or PD – papaya dieback phytoplasma; PYL – *Phormium* yellow leaf phytoplasma; SR – spacer region.

Phormium yellow leaf (PYL), a lethal disease of the large tufted monocotyledons, New Zealand flax (*Phormium tenax*) and mountain flax (*P. cookianum*), was first recognized in New Zealand in 1908 (Boyce and Newhook, 1953). The disease was associated with a phytoplasma following the observation of phytoplasma profiles in the phloem of diseased plants and in its planthopper vector, *Oliarus atkinsoni* (Ushiyama et al., 1969), and a phytoplasma-specific PCR product has consistently been obtained from diseased plants (Andersen et al., 1998). Papaya dieback (PDB or PD), an often devastating disease of papaya (*Carica papaya*) in Queensland, Australia, was first recorded in 1922 (Glennie and Chapman, 1976). Recently, phytoplasmas were detected in PDB affected papaya by PCR (Gibb et al., 1996; Liu et al., 1996; White et al., 1997) but it has not yet been found in diseased plants by electron microscopy. A third disease, Australian grapevine yellows (AGY or AUSGY) was first reported in Australia in 1975. A phytoplasma associated with AGY has been detected by electron microscopy (Magarey et al., 1988) and by PCR (Padovan et al., 1995). No vector of PDB or AGY has yet been identified.

Phytoplasmas belong to the class *Mollicutes*, a diverse group of prokaryotes which evolved from gram-positive bacteria with low G + C content and are characterized by their lack of a cell wall and small genome size. Phytoplasmas have been classified by restriction site and sequence analysis of the 16S rRNA gene (Lee et al., 1993; Schneider et al., 1993; Gundersen et al., 1994; Seemüller et al., 1994) and by sequence analysis of the 16S/23S rRNA SR (spacer region) (Kirkpatrick et al., 1994). A formal taxon-

Table 1. Available sequences of the phytoplasmas used in this study and their accession numbers

Abbreviation	Full name and origin	Region and length of sequence	Accession no.	Reference
AGY	Australian grapevine yellows, Australia	16S rRNA and 16S/23S SR[1], 1708 bp	X95706	Padovan et al., 1996
AUSGY	Australian grapevine yellows, Australia	16S rRNA and 16S/23S SR, 1532 bp	L76865	Davis et al., 1997
PD	Papaya dieback, Australia	16S rRNA, 503 bp	na[2]	White et al., 1997
PD	Papaya dieback, Australia	16S/23S SR, 254 bp	na[2]	White et al., 1997
PDB	Papaya dieback, Australia	16S/23S SR, 227 bp	Y08176	Gibb et al., 1998
PYL	*Phormium* yellow leaf, New Zealand	16S rRNA *rrnA*, 1483 bp	U43569	Liefting et al., 1996
PYL	*Phormium* yellow leaf, New Zealand	16S rRNA *rrnB*, 1483 bp	U43570	Liefting et al., 1996
PYL	*Phormium* yellow leaf, New Zealand	16S/23S SR, 224 bp	U43571	Liefting et al., 1996
STOL	Stolbur of pepper, Serbia	16S rRNA, 1494 bp	X76427	Seemüller et al., 1994
STOL	Stolbur of pepper, Serbia	16S/23S SR, 214 bp	AF035361	Smart et al., in prep
VK	Grapevine yellows, Germany	16S rRNA, 1501 bp	X76428	Seemüller et al., 1994
VK	Grapevine yellows, Germany	16S/23S SR, 214 bp	AF035362	Smart et al., in prep

[1] Spacer region.
[2] Not lodged in the database, sequence obtained from White et al. (1997).

omy for phytoplasmas using the *Candidatus* concept proposed by Murray and Schleifer (1994) was recommended by the International Committee on Systematic Bacteriology Subcommittee on the Taxonomy of *Mollicutes* (Tully, 1995). Under this scheme, each of the major 16S rRNA groups represents a *Candidatus* species of the Phytoplasma genus.

Nucleotide sequences of the 16S rRNA and the 16S/23S SR of the phytoplasmas associated with AGY, PDB and PYL have been reported (references in Table 1). Nucleotide sequence data of AGY obtained directly from PCR products of the 16S rRNA gene and the 16S/23S SR was reported by two independent groups (Padovan et al., 1996; Davis et al., 1997). Likewise, sequence data of the 16S/23S SR from PDB has become available from two independent laboratories, using direct sequencing of PCR products (Gibb et al., 1998; White et al., 1997). White et al. (1997) also sequenced a 503 bp portion of the 16S rRNA gene from PDB. The nucleotide sequence of the 16S rRNA gene of PYL was determined from eight independent PCR-derived clones from four plants (Liefting et al., 1996). Phytoplasmas have been shown to contain two rRNA operons (Schneider and Seemüller, 1994), and the two 16S rRNA genes of PYL phytoplasma were found to differ at four nucleotide positions (Liefting et al., 1996). Nucleotide sequence data available from three independent clones of the 16S/23S SR showed no inter-operon differences (Liefting et al., 1996). Inter-operon differences have not been reported in the sequences of AGY or PDB.

These ribosomal sequences for AGY, PDB and PYL have been used to determine the relationship of these phytoplasmas to other phytoplasmas either by RFLP analysis or sequence comparisons. In all instances, these three phytoplasmas were found to be most closely related to the German grapevine yellows (VK) and stolbur (STOL) phytoplasmas (Gibb et al., 1996; Liefting et al., 1996; Padovan et al., 1996; Davis et al., 1997; White et al., 1997). AGY, PDB, PYL, VK and STOL are all members of subclade xii, designated by Davis et al. (1997). Furthermore, this phylogenetic analysis showed that PYL (Liefting et al., 1996) and AGY (Davis et al., 1997) phytoplasmas formed a separate subgroup from the STOL and VK phytoplasmas within subclade xii. Davis et al. (1997) designated the *Candidatus* species name, '*Candidatus* Phytoplasma australiense', for the phytoplasma associated with AGY.

There have also been some reports of the relationships of AGY, PDB and PYL phytoplasmas to each other. Padovan et al. (1996) reported 99.5% similarity between the 16S rRNA genes of AGY and PYL. AGY was also reported to be very closely related to PDB (Gibb et al., 1996; Padovan et al., 1996). However, there has been no thorough study on the relationship between all three phytoplasmas. In this paper we have evaluated the phylogenetic relationships between AGY, PDB and PYL based on the available sequence data and conclude that all three diseases are associated with the same phytoplasma species.

The 16S rRNA and the 16S/23S SR sequences of the phytoplasmas in Table 1 were separately aligned by the PILEUP algorithm (Feng and Doolittle, 1987) from the GCG 9 (Genetics Computer Group, Wisconsin) software package of Devereaux et al. (1990).

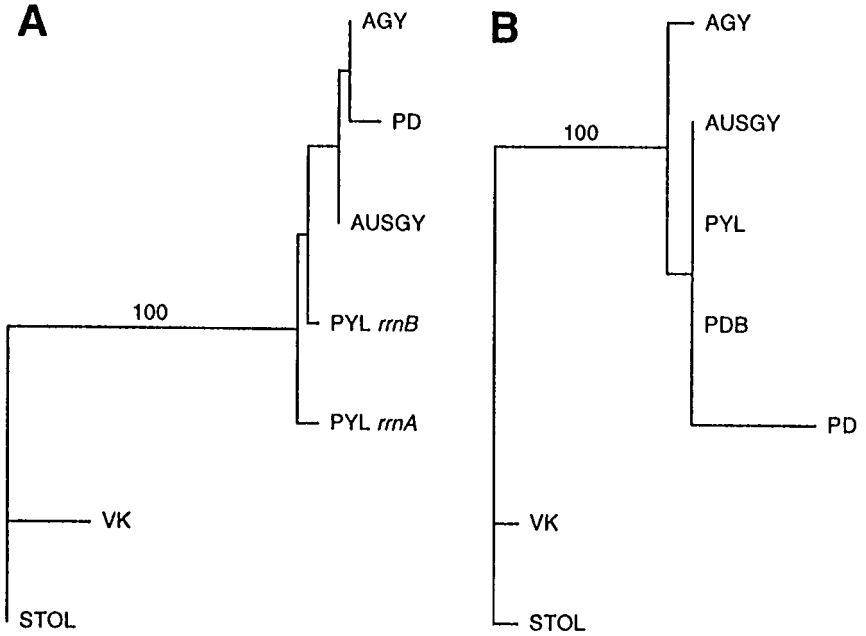

Figure 1. Phylogenetic trees constructed by parsimony analysis comparing (A) 16S rRNA gene sequences, and (B) 16S/23S spacer region sequences of the phytoplasmas in Table 1, using German grapevine yellows (VK) and stolbur (STOL) as the outgroups. Branch lengths are proportional to the number of inferred character state transformations. The numbers above the branches are percent bootstrap values obtained for 1,000 replicates (only values greater than 80% are shown). Abbreviations for phytoplasmas are defined in Table 1.

Nucleotide differences were observed between the two AGY sequences and between the two PDB sequences. There were six differences in the 1,334 nucleotide region of overlap in the 16S rRNA gene between the two AGY sequences. In the 16S/23S SR sequence alignment, the two AGY sequences and the two PDB sequences differed from each other at six and seven positions, respectively. It is possible that the heterogeneity between the two sequences of AGY and the two sequences of PDB represents diversity at the population level between these two phytoplasmas. However, at all of these variable positions, one of the two sequences was identical to all the other sequences in the lineup. Furthermore, no sequence heterogeneity was noted between isolates of PYL from geographically distinct populations of New Zealand flax (Liefting et al., 1996). In view of the known propensity for introducing nucleotide variations using PCR (Saiki et al., 1988; Keohavong and Thilly, 1989) and the fact that some of the variations occur at positions invariant in all other phytoplasmas, it is also possible that at least some of this observed variation may represent artifacts introduced during DNA manipulation. Not withstanding this sequence variation, 16S rRNA sequences from PDB and PYL contain the two signature sequences identified by Davis et al. (1997) for AGY. They are all identical over nucleotides 191-215 and 999-1013 identified as being characteristic for AGY. Therefore, taxonomically PDB and PYL should be considered the same *Candidatus* species as AGY.

Phylogenetic analysis further supported this taxonomic grouping. Maximum parsimony trees were constructed from the aligned sequences using PAUP (phylogenetic analysis using parsimony) version 3.1.1 (Swofford, 1990) as described by Liefting et al. (1996). VK and STOL were used as outgroups. Phylogenetic analysis of the 16S rRNA sequences identified seven equally parsimonious trees (tree length = 47). One of these trees is shown in Figure 1A. Monophyly of the AGY, PDB and PYL phytoplasma clade is well supported by bootstrap (100%) (Felsenstein, 1985). However, none of the groupings among AGY, PDB and PYL were supported (% bootstraps values < 55). The changes between these phytoplasmas may well reflect artifacts as discussed above, but some may reflect inter-operon differences as indicated by the differences between the *rrnA* and *rrnB* 16S rRNA sequences of PYL. Phylogenetic analysis of the 16S/23S SR identified four equally parsimonious trees with one shown in Figure 1B. This tree (tree length = 16) also strongly supports a monophyletic group of AGY, PDB and PYL phytoplasmas. Analysis of the

tuf gene by Schneider et al. (1997) reveals that AGY and PYL exhibit the same RFLP profile after *Sau*3AI digestion, yet differ in their *Hpa*II pattern. Therefore, the phytoplasmas within the '*Candidatus* Phytoplasma australiense' species, as defined by 16S rRNA gene sequences, can be further differentiated by analysis of their *tuf* gene sequences.

The recognition that the same phytoplasma species is associated with AGY, PDB and PYL leads to the question as to its origin. The causal agent of PYL has traditionally been regarded as endemic to New Zealand, a view based on the biology of its hosts and its monophagous vector (Boyce and Newhook, 1953). Both host species (*P. tenax* and *P. cookianum*) and the vector (*O. atkinsoni*) are essentially endemic to New Zealand (*P. tenax* also extends to Norfolk Island), and the disease is only known from New Zealand where it occurs widely in both natural and modified plant communities. Outbreaks of PYL have been associated with habitat disturbances. Disease resistant selections have been recognized (Boyce, 1958), suggestive of the long term association between host and pathogen.

If '*Candidatus* Phytoplasma australiense' is indeed endemic to New Zealand, has the phytoplasma spread from there to Australia or is it also endemic to Australia? AGY and PDB have only to date been identified in Australia from the introduced hosts, grapevines and papaya, respectively. *P. tenax* has been widely cultivated in Australia for more than 150 years and the introduction of the phytoplasma in infected rhizomes from New Zealand is a possibility. However, resolution of the relationships between the New Zealand and Australian populations must await further evidence on the occurrence of this phytoplasma species in other hosts and vectors.

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