Phylogenetic Analysis of “*Candidatus Phytoplasma australiense*” Reveals Distinct Populations in New Zealand

Mark T. Andersen, Richard D. Newcomb, Lia W. Liefting, and Ross E. Beever

First and second authors: HortResearch, Private Bag 92169, Auckland, New Zealand; third author: AgriGenesis Biosciences Ltd., P.O. Box 50, Auckland, New Zealand; and fourth author: Landcare Research, Private Bag 92170, Auckland, New Zealand.
Accepted for publication 10 March 2006.

ABSTRACT

Andersen, M. T., Newcomb, R. D., Liefting, L. W., and Beever, R. E. 2006. Phylogenetic analysis of “*Candidatus Phytoplasma australiense*” reveals distinct populations in New Zealand. *Phytopathology* 96:838-845.

The phytoplasma “*Candidatus Phytoplasma australiense*” has been reported from New Zealand and Australia, where it has been associated with a range of host plants, especially since the 1970s. Partial *tuf* gene sequences of 36 New Zealand (NZ) isolates from four different host genera revealed nine different variants, which clustered into two distinct groups without any obvious correlation with host or geographic region. Phylogenetic analysis of these sequences, together with those available from Australian isolates, revealed three distinct clades: one found solely

in Australia, one found solely in NZ, and a third with representatives from both countries. These divisions are consistent with differences observed in the 16-23S rRNA internal transcribed spacer region; therefore, we conclude that they represent three distinct subgroups: *tuf* 1, *tuf* 2, and *tuf* 3. We estimated a time of divergence for the three clades based on a synonymous substitution rate calculated by comparing the complete *tuf* gene sequence from the Loofah witches'-broom phytoplasma and “*Candidatus Phytoplasma australiense*”. Using a calibration date of 110 million years, the estimated time to a common ancestor for all clades (6 to 9 million years ago) suggests divergence during the Miocene, well after the geological separation of NZ and Australia.

Phytoplasmas are a group of insect-transmitted prokaryotes (class *Mollicutes*) associated with diseases of $\approx 1,000$ plant species (29). They are characterized by the lack of a cell wall, small genome size, and a low G+C content, with none having been cultured in vitro to date (20). Prior to the development of molecular techniques, phytoplasmas were grouped on the basis of biological properties such as symptoms induced, plant host range, and vector specificity. More recently, the focus has been on comparing genetic variation, through both direct DNA sequence comparisons and the use of restriction fragment length polymorphisms (RFLPs). Initial emphasis has been on the 16S rRNA gene (1,22,26), because of its conservation throughout the prokaryotes. Using this approach, more than 14 distinct phylogenetic groups and, in some instances, subgroups of related phytoplasmas have been recognized (17,20,41). Although delimited primarily on the basis of 16S rDNA similarity, other phylogenetic markers, such as the 23S rRNA gene, the 16S-23S rRNA internal transcribed spacer (ITS) regions, and various protein encoding genes, support and, in some cases, extend these groupings (20). The obligate nature of phytoplasmas has hampered formal taxonomic recognition of their variation; however, many of the groups and subgroups are now being recognized as species using the *Candidatus* concept proposed for unculturable organisms (30). This system groups phytoplasmas into *Candidatus* spp. on the basis of 16S rRNA sequence similarity. In general, those isolates that are $\geq 97.5\%$ identical are grouped together in the same *Candidatus* sp. unless significant differences in biological or genetic properties suggest that they should be classified separately (17).

With the exception of “*Candidatus Phytoplasma asteris*” (21), variation within these *Candidatus* spp. has been poorly studied.

However, such variation will give clues to host specificity, ecological history, and genetic structuring. Although the 16S rRNA gene can be useful for delineating variation within species, allowing, for example, recognition of 15 subgroups within “*Ca. P. asteris*” (21), generally it has insufficient variability to reveal intraspecific groupings. The 16S-23S rDNA spacer region is more variable, and can be useful in distinguishing closely related groups of phytoplasmas (42). However, the two rRNA operons in phytoplasmas can be different, making it difficult to distinguish variation between paralogous operons within an isolate from orthologous genes between isolates (23,38). Alternatives include protein coding genes such as ribosomal protein genes (16) and the *tuf* gene, which encodes the elongation factor Tu (EF-Tu) (36). In *Mollicutes*, including phytoplasmas, the *tuf* gene is present as a single copy and can be sequenced readily using a polymerase chain reaction (PCR) approach (6,19).

“*Candidatus Phytoplasma australiense*” is a phytoplasma associated with diseases in New Zealand (NZ) and Australia (11, 24). In NZ, it is associated with diseases of both native and exotic plants, including Phormium yellow leaf (PYL) (25), strawberry lethal yellows (SLY) (3), Cordyline sudden decline (CSD) (2), and Coprosma lethal decline (CLD) (5). In Australia, it is associated with the diseases Australian grapevine yellows (AGY or AUSGY) and papaya dieback (PDB), as well as SLY and strawberry green petal (SGP) (15,27,32,48). The 16S rRNA genes in all these examples are at least 99% similar to each other, indicating that, under the *Candidatus* system, they form a single species (25). Additionally, phytoplasma has been detected using PCR-RFLP analysis in an assortment of other plants in Australia, including *Phaseolus vulgaris* (37), *Catharanthus roseus* periwinkle (12), cottonbush, mung bean, and pumpkin (43), and the Australian native shrubs *Exocarpus cupressiformis* and *Jacksonia scoparia* (44). Sequence differences have been detected between isolates of “*Candidatus P. australiense*” when other genomic regions, such as the *tuf* gene (36), the 16-23S spacer region (32), and the ribosomal protein-encoding (*rp*) operon (43), have

Corresponding author: M. T. Andersen
E-mail address: mandersen@hortresearch.co.nz

been examined. In the present study, we investigated variation in the *tuf* gene in a diverse sample of NZ isolates of “*Ca. P. australiense*” to better understand intraspecific variation and to explore possible association of particular variants with host range and geographic distribution, especially in relation to Australian populations.

MATERIALS AND METHODS

Plant material. Plants showing symptoms of known phytoplasma diseases were sampled from field locations throughout NZ. They consisted of plants from the following four genera, with the number of samples indicated: *Coprosma robusta* ($n = 6$), *Cordyline australis* ($n = 4$), *C. banksii* ($n = 1$), *Phormium tenax* ($n = 12$), and strawberry ($n = 14$) (Table 1). DNA was extracted from 0.3 g of tissue according to the protocol of Gardiner et al. (14) and resuspended in 100 μ l of H₂O. DNA was further purified using S300HR, a size-exclusion spin column, as specified by the manufacturer (Amersham Biosciences, UK).

Gene amplification and sequencing. Template DNA (10 to 50 ng) was amplified as described previously (2), in either a Corbett FTS-1S or FTS-4000 Thermal Sequencer with the following conditions: 94°C for 2 min, 53°C for 1 min, 72°C for 2 min for 1 cycle, then 94°C for 1 min, 53°C for 30 s, and 72°C for 2 min for 35 cycles. Reaction conditions were 20 mM Tris-Cl (pH 8.4), 50 mM KCl, 200 μ M dNTPs, 1.5 mM MgCl₂, and 0.5 unit of *Taq* polymerase (Invitrogen, Carlsbad, CA), with a final primer concentration of 100 nM.

Fragments (949 bp in length) of “*Ca. P. australiense*” *tuf* gene were generated using primers fTuf AY and rTuf AY (36). Two further primers, Tuf1FA (5'-GATAGTGTAATGCCTCA-3') and Tuf4R (5'-CCTGCTTGAGCAAAATCTA-3') were designed to be used in nested PCR from the fTufAY/rTufAY amplicon. Four primers (two forward and two reverse), designed to distinguish between “*Ca. P. australiense*” tuf 1 and tuf 2, are listed in Table 2.

PCR products of the 16-23S spacer region of the rRNA operon were generated using primers P1 (13) and P7 (39). Ambiguities created by insertions and deletions were resolved by sequencing from cloned fragments. The P1/P7 PCR fragment of NZSb11 was cloned into pGEM-T easy (Promega Corporation, Madison, WI) according to the manufacturer's specifications, and DNA was prepared using the QIAGEN Plasmid miniprep kit according to their protocols (QIAGEN GmbH).

DNA sequencing was conducted using an ABI Prism 377 (PE Applied Biosystems, Foster City, CA) with Big Dye Terminator chemistry. Unless stated otherwise, PCR products were sequenced, with primers and salts removed by passing samples through an S300HR size-exclusion column (Amersham Biosciences). Primers used for sequencing were fTufAY, rTufAY, Tuf1FA, Tuf4R for the *tuf* gene, NP6AF (23), and P7 for the 16-23S SR. Universal sequencing primers SP6 and T7 were used for further sequencing of cloned fragments. All isolates were sequenced at least twice from separate PCR reactions. No differences between sequences of each isolate were observed.

Database sequences. GenBank accession numbers of *tuf* gene sequences used for comparison are Loofah witches'-broom

TABLE 1. Isolates of “*Candidatus Phytoplasma australiense*” with host plant, geographical location, *tuf* gene variation, and GenBank accession number^a

Isolate	Host plant	Geographical location	<i>Tuf</i> variant group	<i>Tuf</i> gene clade	Accession number
NZPh1	<i>Phormium tenax</i>	Auckland	I	2	DQ094772
NZPh2	<i>Phormium tenax</i>	Auckland	I	2	...
NZPh3	<i>Phormium tenax</i>	Northland	II	2	DQ094773
NZPh4	<i>Phormium tenax</i>	Northland	II	2	...
NZPh5	<i>Phormium tenax</i>	Bay of Plenty	III	2	DQ094774
NZPh6	<i>Phormium tenax</i>	Northland	IV	2	DQ094775
NZPh7	<i>Phormium tenax</i>	Nelson	V	2	DQ094776
NZPh8	<i>Phormium tenax</i>	Nelson	V	2	...
NZPh9	<i>Phormium tenax</i>	Wellington	VI	2	DQ094777
NZPh10	<i>Phormium tenax</i>	Taupo	VI	2	...
NZSb7	Strawberry cv. Pajaro	Bay of Plenty	VII	2	DQ094778
NZPh11	<i>Phormium tenax</i>	Coromandel	VII	2	...
NZSb1	Strawberry cv. Chandler	Bay of Plenty	VII	2	...
NZSb2	Strawberry cv. Pajaro	Waikato	VII	2	...
NZSb3	Strawberry cv. Pajaro	Waikato	VII	2	...
NZSb4	Strawberry cv. Camarosa	Bay of Plenty	VII	2	...
NZSb5	Strawberry cv. Pajaro	Waikato	VII	2	...
NZSb6	Strawberry cv. Camarosa	Bay of Plenty	VII	2	...
NZSb8	Strawberry cv. Camarosa	Bay of Plenty	VII	2	...
NZCp1	<i>Coprosma robusta</i>	Auckland	VII	2	...
NZCp2	<i>Coprosma robusta</i>	Auckland	VII	2	...
NZCp3	<i>Coprosma robusta</i>	Auckland	VII	2	...
NZCp4	<i>Coprosma robusta</i>	Auckland	VII	2	...
NZCp5	<i>Coprosma robusta</i>	Auckland	VII	2	...
NZCt1	<i>Cordyline australis</i>	Auckland	VII	2	...
NZCt2	<i>Cordyline australis</i>	Auckland	VII	2	...
NZCt3	<i>Cordyline australis</i>	Auckland	VII	2	...
NZSb9	Strawberry cv. Seascape	Bay of Plenty	VIII	1	DQ094779
NZSb10	Strawberry cv. Pajaro	Waikato	VIII	1	...
NZCt4	<i>Cordyline banksii</i>	Auckland	IX	1	DQ094780
NZPh12	<i>Phormium tenax</i>	Bay of Plenty	IX	1	...
NZCp6 ^b	<i>Coprosma robusta</i>	Bay of Plenty	IX	1	...
NZCt5	<i>Cordyline australis</i>	Auckland	IX	1	...
NZSb11	Strawberry cv. Pajaro	Bay of Plenty	IX	1	...
NZSb12	Strawberry cv. Camarosa	Bay of Plenty	IX	1	...
NZSb13	Strawberry cv. Selva	Bay of Plenty	IX	1	...
NZSb14	Strawberry cv. Pajaro	Bay of Plenty	IX	1	...

^a Geographical location was according to the scheme as described by Crosby et al. (9). Only one example of each was submitted to GenBank because all members of a variant group are identical.

^b Isolate NZCp6 could be detected only after nested polymerase chain reaction with primers fTufAY and rTufAY followed by Tuf1FA and Tuf4R. The sequence of amplified DNA contained polymorphisms at positions 357 to 774 identical to those in variant group IX.

(AF086617), SLY (AY303552), CBWB (AY303553), CBRYL (AY303554), PYLC (AY303555), SLY1 (AY303556), AGY (AY303557), SGP (AY303571), KV (L46369), and STOLF (L46370). NZ samples sequenced here were: NZPh1 (DQ094772), NZPh3 (DQ094773), NZPh5 (DQ094774), NZPh6 (DQ094775), NZPh7 (DQ094776), NZPh9 (DQ094777), NZCt4 (DQ094780), NZSb7 (DQ094778), and NZSb9 (DQ094779). The complete sequence of “*Ca. P. australiense*” *tuf* gene (DQ096804) was obtained from isolate NZSb11 as part of a “*Ca. P. australiense*” genome sequencing project. Sequences of the 16S-23S rRNA spacer region deposited in GenBank (with accession numbers) are NZPh6 (DQ286404), NZPh2 (DQ286405), NZPh4a (DQ286406), NZPh4b (DQ286407), NZCt1a (DQ286408), NZCt1b (DQ286409), NZSb10a (DQ286410), NZSb11b (DQ286411), NZSb10b (DQ286412), and NZSb11a (DQ286413). Suffixes “a” and “b” refer to the different operons from the same isolate. Ambiguity codes are given for positions where interoperon differences were not able to be resolved. 16S rRNA sequences used for comparison were PYL *rrnA* (U43569), PYL *rrnB* (U43570), PDB (Y10095), SGP (AJ243044), SLY (AJ243045), and AGY (X95706).

Sequence analysis. Sequences were analyzed using Sequencher (version 4.0.5; Gene Codes Corporation, Ann Arbor MI) and Vector NTI 9.0.0 InforMax (Invitrogen), and aligned using Clustal_X (46). Phylogenetic trees were inferred using parsimony as implemented within PAUP* (45). Rates of change in synonymous (K_S) and nonsynonymous (K_A) positions were calculated using the method of Nei and Gojobori (31) as implemented in DnaSP Version 4.0 (34).

RESULTS

The complete *tuf* gene sequence and estimation of divergence times. The complete sequence of the *tuf* gene (DQ096804) of isolate NZSb11 is 1,185 nucleotides in length, with a G+C content of 36.5%. The predicted gene product of 394 amino acids

contains the three structural domains of elongation factor Tu (NCBI Conserved Domain Search). A BLASTP search of the non-redundant database at NCBI showed that this gene shared high homology with the *tuf* gene from onion yellows (NC 005303, 95% amino acid identity) and aster yellows witches'-broom (AY277404, 94% identity) phytoplasmas. The degree of homology was significantly lower with apple proliferation (APR011104, 71% identity) and loofah witches'-broom (AF086617, 61% identity) phytoplasmas.

A substitution rate for the phytoplasma *tuf* gene was calculated by comparing the full-length *tuf* gene sequence of the Loofah witches'-broom phytoplasma with that of “*Ca. P. australiense*”, two phytoplasmas that reflect the maximum divergence of the genus “*Candidatus* Phytoplasma” (41). From this, the average number of substitutions per synonymous sites (K_S) was estimated at 0.5465 (147/269) substitutions per position. Based on the rate of change of the 16S rRNA gene, it has been proposed that the Phytoplasma clade arose approximately 180 million years ago (MYA), and that the time to the common ancestor for all phytoplasmas was ≈ 110 MYA (28). Using this estimate, we calculate the evolutionary rate of the phytoplasma *tuf* gene to be 5.0×10^{-9} substitutions per position per year.

***Tuf* gene sequences.** PCR fragments of the *tuf* gene from 36 isolates of “*Ca. P. australiense*” were generated by primers fTuf AY and rTuf AY, sequenced, and compared. Nineteen polymorphic sites were detected over an 815-bp region (between nucleotide positions 195 to 1009, based on the complete gene sequence). The sequence of the *tuf* gene from the 36 isolates could be grouped into nine different variants, I to IX (Table 3). The nine variants clustered into two distinct groups, which differ at 10 or more of the 19 variable sites. The greatest difference between any two samples was at 16 positions, corresponding to a maximum divergence of 2.0% at a nucleic acid level. Considering the NZ isolates, the predicted amino acid sequences of variants VII and VIII were identical over the sequenced region. The other variants differed by one (variants III, IV, V, VI, and IX) or two (variants I and II) amino acids from the predicted consensus amino acid sequence. The maximum divergence at an amino acid level among all NZ samples was 1.1% (3/272).

A phylogenetic tree of the “*Ca. P. australiense*” *tuf* gene nucleotide sequences was created using the 36 samples from NZ augmented with 7 from Australia (44). The sequences separated into three distinct clades (Fig. 1). Clade 1 (including variants VIII and IX) contains isolates from both countries, clade 2 (including

TABLE 2. Primers designed to distinguish the *tuf* gene clades 1 and 2 of “*Candidatus* Phytoplasma australiense”

	Clade	Position	Sequence 5'–3'
PaTC1F	1	306–324	CATTCTAGTTGTTTCTGGT
PaTC1R	1	858–843	GTCTTCTCGGTTAATC
PaTC2F	2	306–324	TATTTTAGTTGTTTCTGGA
PaTC2R	2	858–843	GTCTTCTCGGTTAATA

TABLE 3. Nucleotide polymorphisms in the *tuf* gene of “*Candidatus* Phytoplasma australiense” in isolates from Australia and NZ (variant groups I to IX) between positions 195 and 1008^a

Group	Nucleotide positions																														
	1	2	3	3	3	3	3	3	3	3	4	5	5	5	5	6	6	6	6	7	7	8	8	8	8	8	8	8	9	9	0
	9	7	0	1	2	2	3	5	6	7	9	0	3	5	6	1	1	2	6	1	7	2	3	4	4	5	8	3	5	0	
5	9	6	0	4	5	6	7	9	8	5	1	2	6	0	2	9	8	6	2	4	2	0	0	3	2	8	9	1	8		
I	T	T	T	T	A	G	A	C	T	A	T	C	G	A	C	A	G	C	C	A	G	C	G	C	T	C	A	T	T	T	
II	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	G	.	.	.	.	.	.	.	T	.	.	.	.	.	.	.
III	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	G	.	G	.	.	.	.	.	T	.	.	.	.	.	.	.
IV	.	.	.	.	.	T	.	.	.	.	.	.	.	.	.	G	.	G	.	.	.	.	.	C	T	.	.	.	.	.	.
V	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	G	.	G	T	.	.	.	.	C	T	.	.	.	.	.	.
VI	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	G	.	G	.	A	.	.	.	C	T	.	.	.	.	.	.
VII	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	G	.	G	.	.	.	.	.	C	T	.	.	.	.	.	.
VIII	.	.	C	C	T	.	.	T	.	T	.	.	.	.	.	G	.	T	.	.	T	A	.	C	T	G	A	.	.	C	.
IX	.	.	C	C	T	.	.	T	.	T	.	.	.	.	.	G	.	T	.	.	T	G	A	.	C	T	G	A	.	C	C
AGY	?	?	?	?	?	.	T	T	C	T	.	.	.	.	.	G	.	T	.	.	T	.	?	?	?	?	?	?	?	?	?
SGP	.	.	C	C	T	.	.	T	.	T	.	.	.	.	.	G	.	T	.	.	T	.	A	.	C	T	G	A	.	C	C
SLY1	.	.	C	C	T	.	.	T	.	T	.	.	.	.	.	G	.	T	.	.	T	.	A	.	C	T	G	A	.	C	C
PYCL	C	.	C	C	T	.	.	T	.	T	.	.	.	.	.	G	.	T	.	.	T	.	A	.	C	T	G	A	.	C	C
CBWB	.	.	C	C	T	.	.	T	.	T	.	.	.	.	.	G	.	T	.	.	T	.	A	.	C	T	G	A	.	C	C
CBRYL	.	C	.	.	C	.	.	.	.	.	C	A	A	G	A	G	.	.	T	.	A	T	C	T	G	T	G	.	C	C	
SLY	.	C	.	.	C	.	.	.	.	.	C	A	A	G	A	G	.	.	T	.	A	T	C	T	G	T	G	.	C	C	

^a Nucleotide positions are in accordance with the complete gene sequence of “*Ca. P. australiense*” (DQ096804). Only polymorphic sites are shown. A dot indicates no difference from variant group I (top row) and missing data is indicated with a question mark (?).

variants I to VII) consists exclusively of isolates from NZ, and clade 3 comprises two representatives only, both from Australia.

No simple relationship is apparent between variants and host or geographical location (Table 4). Variants I to VI (clade 2) are only reported from naturally growing stands of *Phormium* spp., and each was found at only one or two widespread localities. Variant VII (clade 2) was found in all hosts, with all samples within the Auckland-Coromandel and nearby Bay of Plenty-Waikato re-

gions, within an approximately circular area of radius 80 km. The sample from *Phormium* spp. was from a natural stand, those from *Coprosma* and *Cordyline* spp. were from restoration plantings, and those from strawberry from intensively managed beds. Both clade 1 variants also were reported from this same general area, with variant VIII reported only from strawberry beds and variant IX from naturally growing *Phormium*, *Coprosma*, and *Cordyline* spp. as well as strawberry.

Fig. 1. Phylogenetic tree constructed by parsimony as implemented within PAUP* (45) of near full-length *tuf* gene sequences of “*Candidatus Phytoplasma australiense*” from Australia and New Zealand. New Zealand samples (prefixed with “NZ”) consist of 815-bp fragments, as follows: NZPh1, NZPh2, NZPh3, NZPh4, NZPh5, NZPh6, NZPh7, NZPh8, NZPh9, NZPh10, NZPh11, NZPh12, NZCp1, NZCp2, NZCp3, NZCp4, NZCp5, NZCt1, NZCt2, NZCt3, NZCt4, NZCt5, NZSb1, NZSb2, NZSb3, NZSb4, NZSb5, NZSb6, NZSb7, NZSb8, NZSb9, NZSb10, NZSb11, NZSb12, NZSb13, and NZSb14 (accession numbers, provided in Table 1). Australian samples AGY, Australian grapevine yellows; SGP, strawberry green petal; SLY1, strawberry lethal yellows; PYLC, pumpkin yellow leaf curl; CBWB, cottonbush witches’-broom; and CBRYL, cottonbush reduced yellow leaves were obtained from GenBank. STOLF, Stolbur and KV, Clover phyllody were used as outgroups (for accession numbers, see Materials and Methods). The tree is one of 228 shortest trees of length 183. Bootstrap values generated from 1,000 bootstrap replicates are shown under branches or with arrows.

Tuf clade 1- and 2-specific PCR. Using the sequence data, four primers (two forward and two reverse) were designed to distinguish tuf clade 1 from tuf clade 2. The primers were tested on 11 samples (NZPh2, NZPh4, NZPh6, NZPh9, NZPh10, NZSb1, NZCt1, NZSb10, NZPh12, NZCt5, and NZSb11) and confirmed the classification as determined by sequence. All samples gave appropriate size bands for the designated primers. No sample gave bands for both types, suggesting the absence of mixed infections of tuf clade 1 and tuf clade 2 phytoplasmas.

Molecular clock analysis. We calculated the time of divergence of the different clades based on our estimated substitution rate (Table 5). Estimates were 9 MYA for the common ancestor of tuf clade 1 and tuf clade 2, 6 MYA for the ancestor of tuf clade 2 and tuf clade 3, and 7 MYA for the ancestor of tuf clade 1 and tuf clade 3. The time to common ancestor for the tuf clade 2 was estimated at ≈ 2 MYA, and for tuf clade 1 at ≈ 0 MYA, reflecting the limited variation in the latter clade.

16-23S ITS region. The ITS regions of 10 isolates were sequenced and compared to establish whether variations in this region reflect the phylogeny observed for the *tuf* gene. Of approximately 12 polymorphic sites identified among isolates, only 5 were informative with respect to phylogeny. These five positions consisted of single base insertions or deletions (INDELS) linked to small mononucleotide tracks, and were associated with either tuf clade 1 or tuf clade 2. All INDELS occurred outside the tRNA^{Leu} that lies between positions 1576 and 1652. Five distinct types (A to E) were identified when INDELS were used as distinguishing criteria (Table 6). All those belonging to tuf clade 2 (seven samples) were homogeneous with respect to the five INDELS, whereas all tuf clade 1 (three isolates) were heterogeneous (Table 7). Isolate NZCt1 differed from all other isolates at positions 1691 and 1740.

DISCUSSION

This study presents a comprehensive survey of sequence variation of the *tuf* gene from NZ isolates of “*Ca. P. australiense*”. These isolates fall into two distinct clades, each comprising a number of variants. Although the 16S-23S spacer region showed less variation between isolates compared with the *tuf* gene, the groupings that the spacer region reveals were consistent with most variants in tuf clade 2 being identical and variants VIII and IX in tuf clade 1 differing from these and from each other (Table 7). The only exception was variant VII, where isolate NZCt1 differed from all other samples analyzed. Coupled with evidence from other phytoplasmas that *tuf* gene variation usually matches 16S rRNA variation but provides greater resolution (40), we conclude that these two *tuf* gene clades reflect two subspecific taxonomic groups present in NZ. In conformity with present usage (20,40), we refer to these as subgroups.

This conclusion supports and extends results involving mainly Australian isolates. Schneider et al. (36) first recognized variation

in the *tuf* gene in “*Ca. P. australiense*” by RFLP analysis using the restriction enzyme *Hpa*II to distinguish between a grape (AGY) sample from Australia from a *Phormium* sp. (PYL) sample from NZ. More recently, Streten and Gibb (43) extended this work, recognizing three subgroups based primarily on *tuf* gene variation, and our findings confirm this conclusion. Their subgroup ‘tuf-Australia I’ included isolates from pumpkin, papaya, grapevine, *Gomphocarpus* spp., and strawberry in Australia, their ‘tuf-New Zealand I’ isolates from strawberry and *Gomphocarpus* in Australia, and their ‘tuf-New Zealand II’ an isolate from *Phormium* spp. in New Zealand. RFLP analysis of the ribosomal protein gene operon in isolates from their tuf-Australia I and tuf-New Zealand I supported the recognition of these subgroups and also identified a further possible subgroup represented by an isolate from mung bean, the only isolate in the study from Western Australia (43). However, no *tuf* gene or 16S rRNA data has been presented for this latter isolate and further samples are desirable to substantiate this subgroup. Our phylogenetic analysis includes most of the sequences used in their study. Their tuf-Australia I sequences, designated AGY, SGP, SLY1, PYLC, and CMWB, fall into our tuf clade 1, and their tuf-New Zealand I sequences designated SLY2 and CBRYL comprise our tuf Clade 3. Their single tuf-New Zealand II sequence was our sequence NZPh3 in our tuf clade 2.

Streten and Gibb (43) grouped the two Australian isolates, SLY2 and CBRYL, as tuf-New Zealand I. However we did not find such sequences among our isolates of NZ phytoplasmas. Although these isolates are from Australia, they presumably were named ‘tuf New Zealand’ because they share the same RFLP pattern using restriction enzyme *Hpa*II with isolate NZPh3 (PYL in their study). The PCR fragment generated by primers fTufAY and rTufAY has one *Hpa*II site for those isolates in tuf clade 1 and two *Hpa*II sites in tuf clades 2 and 3. The *Hpa*II sites in tuf clades 2 and 3, however, are not identical, because tuf clade 2 has sites at 491 and 666, whereas tuf clade 3 has sites at 491 and 888. Thus, the predicted fragment sizes are very similar and would be difficult to distinguish by gel electrophoresis. This difficulty

TABLE 5. Estimates of time to common ancestor using the number of synonymous nucleotide changes in the *tuf* gene of “*Candidatus* phytoplasma”

Comparison	Isolate	Time (MYA) ^a
Tuf 1 vs. tuf 1	NZSb11 vs. NZSb9	0.0
Tuf 2 vs. tuf 2	NZSb7 vs. NZPh1	1.86
Tuf 1 vs. tuf 2	NZSb7 vs. NZSb11	9.30
Tuf 1 vs. tuf 3	NZSb11 vs. CBRYL	7.46
Tuf 2 vs. tuf 3	NZSb7 vs. CBRYL	5.60
Tuf 2 vs. outgroup	NZSb7 vs. Stolbur	74.94
Tuf 1 vs. outgroup	NZSb11 vs. Stolbur	73.07
Tuf 3 vs. outgroup	CBRYL vs. Stolbur	66.72
Outgroup vs. outgroup	STOLF vs. KV	84.52

^a MYA = million years ago. Evolutionary rate for phytoplasma *tuf* gene was estimated to be 5.0×10^{-9} nucleotide substitutions per position per year.

TABLE 4. Numbers of New Zealand isolates of “*Candidatus* Phytoplasma australiense” within each *tuf* gene variant group according to plant host and geographic region

	Variant group									Total
	I	II	III	IV	V	VI	VII	VIII	IX	
Host										
<i>Phormium</i> spp.	2	2	1	1	2	2	1	...	1	12
Strawberry	...	...	...	...	...	...	8	2	4	14
<i>Coprosma</i> spp.	...	...	...	...	...	...	5	...	1	6
<i>Cordyline</i> spp.	...	...	...	...	...	...	3	...	2	5
Region										
Northland	...	2	...	1	...	...	...	...	...	3
Auckland/Coromandel	2	...	...	...	...	...	9	...	2	13
BoP/Waikato	...	...	1	...	...	...	8	2	6	17
Taupo/Wellington	...	...	...	...	...	2	...	...	...	2
Nelson	...	...	...	...	2	...	...	...	...	2

could be overcome for rapid identification of isolates by PCR using clade-specific primers, as we have demonstrated for tuf clades 1 and 2 (Table 2), with the added advantage that it would allow ready detection of mixed infections. Sequence analysis of the tuf clade 3 isolates indicates that they will not be amplified by the proposed combinations of primers. The subgroup terminology introduced by Streten and Gibb (43) is confusing because, as discussed, their tuf-Australia I clade now includes NZ isolates, and no tuf-New Zealand I subgroup isolates have been found in NZ. Thus, we suggest referring to the subgroups as 'tuf 1', 'tuf 2', and 'tuf 3'.

Subgroups tuf 1 and tuf 2 can be distinguished by diagnostic PCR (Table 2). The variation we observed within tuf 1 (variant groups VIII and IX) and tuf 2 (variant groups I and VII) was minimal compared with the variation between the clades and does not compromise the use of the diagnostic primers described here.

Phylogeographic analysis is a powerful approach to understanding biogeographic distribution patterns (4), but has been little used with bacteria because many taxa presumably are dispersed readily worldwide. However, host-restricted groups such as the rhizobia (33) and the mollicutes offer opportunities for this approach. We estimated that the evolutionary rate for the tuf gene of phytoplasmas (based on synonymous substitutions) was 5.0×10^{-9} substitutions per position per year. This rate is comparable to the proposed tuf gene evolutionary rate for the genus *Buchnera*, bacterial endosymbionts of aphids, which was estimated to be between 3.9 to 8.0×10^{-9} (7). Both rates are significantly higher than for free-living bacteria such as *Escherichia coli* and *Salmonella typhimurium* (7). Reasons advanced for such differences include the absence of selective codon preference, the influence of Muller's ratchet (7,8), and the higher mutation rate for endocellular symbionts (17). That the evolutionary rate for the tuf gene of "Ca. Phytoplasma" appears similar to that of *Buchnera* spp. is not entirely surprising. Phytoplasmas share a number of features with *Buchnera* spp., including small, A+T-rich genomes as a result of genome reduction from free-living ancestors. Both genera comprise obligate intracellular parasites or symbionts of insects, although phytoplasmas are also parasites of plants. Furthermore, both consist of small populations with limited ability to correct genetic errors through heterologous or homologous recombination, and with significant bottlenecks in their respective lifecycles. Although these similarities are a result of convergent evolution, they are the conditions that will result in increased fixation of mutations by the process of Muller's Ratchet (7,47).

Previously, we have suggested that "Ca. P. australiense" is indigenous to NZ, based on its widespread occurrence in that country in naturally growing stands of the endemic host *Phormium* spp., its early reported occurrence as PYL disease in 1908, its transmission by the endemic planthopper *Oliarus atkinsoni*, and the existence in the *Phormium* spp. population of genetically based disease resistance (25). The widespread but localized distribution of tuf variants I to VI (Tables 1 and 4), which presently are known only from *Phormium* spp., is supportive of this conclusion. However, although we have suggested previously that "Ca.

P. australiense" may have been transported from NZ to Australia in infected rhizomes of *Phormium* spp. prior to the emergence of associated diseases there (25), our present findings indicate a more complex historical scenario. The basal position of the subgroup tuf 3, which contains only Australian isolates (Fig. 1), indicates that the species "Ca. P. australiense" may have arisen in Australia, ultimately from an ancestor in the stolbur group. Although our molecular clock estimates undoubtedly are imprecise, and limited by the number and diversity of isolates examined, they indicate that the common ancestor of tuf 1 and tuf 2 diverged from tuf 3 perhaps 6 to 9 MYA, during the Miocene, clearly well after the separation of NZ from Australia, which occurred ≈ 80 MYA in the Cretaceous (35). Subgroup tuf 2, which comprises only NZ isolates, may reflect an ancient long-distance dispersal event from Australia to NZ, perhaps involving wind dispersal of an infected insect vector. The divergence within this subgroup indicates that these isolates shared a common ancestor 1 to 2 MYA in the Pliocene to Pleistocene, indicating that this dispersal event probably took place before this time. The origins of subgroup tuf 1 are more debatable. Although none of the NZ isolates match those from Australia exactly, we suggest that the presence of isolates of this clade in both countries reflects at least one recent dispersal event. If the common ancestor of subgroups tuf 1 and tuf 2 dispersed to NZ prior to their divergence, then this dispersal would be consistent with the proposal of recent, human-mediated movement in *Phormium* rhizomes from NZ to Australia. On the other hand, if the divergence of subgroup tuf 1 from the common ancestor occurred in Australia, a scenario consistent with the early dispersal of subgroup tuf 2, then our findings are consistent with recent potentially human-mediated dispersal from Australia to NZ. This could have occurred, for example, through the exchange of infected strawberry or other plants prior to the imposition of modern quarantine procedures. Clarification of the occurrence of "Ca. P. australiense" infecting native plant hosts in Australia will help distinguish these hypotheses.

It was anticipated that study of phytoplasma variation might help illuminate reasons for the apparent emergence of new diseases in NZ since the 1970s. Both SLY, first recognized in the late

TABLE 7. Correlation between 16-23S rRNA spacer region and tuf types

Isolate	Tuf variant group	Tuf gene clade	SR type ^a
NZPh2	I	2	A-A
NZPh4	II	2	A-A
...	III	2	ND
NZPh6	IV	2	A-A
...	V	2	ND
NZPh9, NZPh10	VI	2	A-A
NZSb1	VII	2	A-A
NZCt1	VII	2	B-B
NZSb10	VIII	1	C-D
NZPh12, NZSb11	IX	1	D-E

^a The two spacer region (SR) operons of all tuf 2 samples were homogeneous for insertion and deletions, whereas those for the tuf 1 isolates were heterogeneous; ND = not determined.

TABLE 6. Positions of insertion and deletions (INDELS) in the 16-23S rRNA spacer region (SR)^a

SR type	Nucleotide positions of INDELS in mononucleotide tracks.					Representative isolates
	1536	1583	1696	1707	1756	
A	—	—	—	T	G	NZPh2
B	—	—	—	—	—	NZCt1
C	—	—	A	T	G	NZSb10b
D	—	T	A	T	G	NZSb11b
E	A	—	A	T	G	NZSb11a

^a Five different sequences were identified and are labeled A to E. All INDELS are associated with short mononucleotide tracks. Positions are arbitrarily assigned to the 5' end of the mononucleotide run as they relate to the sequence of SR type A. These positions will vary for the other types in accordance with insertions and deletions. Nucleotide positions are based on the first base of the 16S rRNA sequence of *Oenothera* aster yellows (M30790) being position 1. Dash (—) indicates absence of a nucleotide at INDEL site.

1970s (49), and CSD, first recognized in the 1980s (2), are conspicuous diseases unlikely to have been overlooked much prior to these years. On the other hand, CLD is more inconspicuous, but there are indications it also emerged in the 1970s, and we have proposed that infected *Coprosma robusta* acts as an infection source for the diseases of strawberry and *Cordylina* spp., which probably are terminal hosts that do not act as sources for further vector infection (5). The association of these “new” diseases with only three of the nine *tuf* variants recognized here provides some support for this proposal. Variants VII and IX both have been found in *Coprosma* and *Cordylina* spp. and strawberry, as well as *Phormium* spp., in the same geographic area (Table 4), and the third variant, variant VIII, is known only from two strawberry samples also in this area. The only known vector of “*Ca. P. australiense*” is *O. atkinsoni*, which largely is restricted to *Phormium* spp. (10,24). This suggests that at least one other vector is associated with transmission to other hosts (5). Although *O. atkinsoni* occasionally may feed on other plant species, thereby transmitting variants from *Phormium* spp. to other hosts such as *Coprosma* spp., widespread dissemination from such a “new” host is likely due to this second, presumably polyphagous, vector. Such a hypothesis implies that variants VII, VIII, and IX originate in *Phormium* spp. Although this is plausible for variant VII, as part of the NZ-restricted *tuf* 2, it is not necessarily so for variants VIII and IX, especially if they have arrived recently in NZ from Australia. A better understanding of the ecology of these diseases must await identification of the insect vector or vectors associated with transmission.

ACKNOWLEDGMENTS

Funds for this research were provided by the New Zealand Foundation for Research, Science and Technology. The complete sequence of “*Ca. P. australiense*” *tuf* gene was obtained as part of a “*Ca. P. australiense*” genome sequencing project (Marsden Fund, Royal Society of New Zealand, Contract No. GRD001).

LITERATURE CITED

- Ahrens, U., and Seemüller, E. 1992. Detection of DNA of plant pathogenic mycoplasma-like organisms by a polymerase chain reaction that amplifies a sequence of the 16S rRNA gene. *Phytopathology* 82:828-832.
- Andersen, M. T., Beever, R. E., Sutherland, P. W., and Forster, R. L. S. 2001. Association of ‘*Candidatus* Phytoplasma australiense’ with sudden decline of cabbage tree in New Zealand. *Plant Dis.* 85:462-469.
- Andersen, M. T., Longmore, J. L., Liefting, L. W., Wood, G. A., Sutherland, P. W., Beck, D. L., and Forster, R. L. S. 1998. *Phormium* yellow leaf phytoplasma is associated with strawberry lethal yellows disease in New Zealand. *Plant Dis.* 82:606-609.
- Avice, J. C. 2000. *Phylogeography: The History and Formation of Species*. Harvard University Press, Cambridge, MA.
- Beever, R. E., Wood, G. A., Andersen, M. T., Pennycook, S. R., Sutherland, P. W., and Forster, R. L. S. 2004. ‘*Candidatus* Phytoplasma australiense’ in *Coprosma robusta* in New Zealand. *N.Z. J. Bot.* 42:663-675.
- Berg, M., and Seemüller, E. 1999. Chromosomal organization and nucleotide sequence of the genes coding for the elongation factors G and Tu of the apple proliferation phytoplasma. *Gene* 226:103-109.
- Brynnel, E. U., Kurland, C. G., Moran, N. A., and Andersson, S. G. E. 1998. Evolutionary rates for *tuf* genes in endosymbionts of aphids. *Mol. Biol. Evol.* 15:574-582.
- Canbäck, B., Tamas, I., and Andersson, S. G. E. 2004. A phylogenomic study of endosymbiotic bacteria. *Mol. Biol. Evol.* 21:1110-1122.
- Crosby, T. K., Dugdale, J. S., and Watt, J. C. 1998. Area codes for recording specimen localities in the New Zealand subregion. *N.Z. J. Zool.* 25:175-183.
- Cumber, R. A. 1953. Investigations into yellow-leaf disease of *Phormium*. IV. Experimental induction of yellow-leaf condition in *Phormium tenax* Forst. by the insect vector *Oliarus atkinsoni* Myers (Hem., cixiidae). *N. Z. J. Sci. Technol.* 34:31-40.
- Davis, R. E., Dally, E. L., Gundersen, D. E., Lee, I. M., and Habili, N. 1997. “*Candidatus* phytoplasma australiense,” a new phytoplasma taxon associated with Australian grapevine yellows. *Int. J. Syst. Bacteriol.* 47:262-269.
- Davis, R. E., Jacobson, S. C., De La Rue, S. J., Tran-Nguyen, L., Gunua, T. G., and Rahamman, S. 2003. Phytoplasma disease surveys in the extreme north of Queensland, Australia, and the island of New Guinea. *Australas. Plant Pathol.* 32:269-277.
- Deng, S., and Hiruki, C. 1991. Amplification of 16S rRNA genes from culturable and nonculturable Mollicutes. *J. Microbiol. Methods* 14:53-61.
- Gardiner, S. E., Bassett, H. C. M., Noiton, D. A. M., Bus, V. G., Hofstee, M. E., White, A. G., Ball, R. D., Forster, R. L. S., and Rikkerink, E. H. A. 1996. A detailed linkage map around an apple scab resistance gene demonstrates that two disease resistance classes both carry the *Vf* gene. *Theor. Appl. Genet.* 93:485-493.
- Gibb, K. S., Persley, D. M., Schneider, B., and Thomas, J. E. 1996. Phytoplasmas associated with papaya diseases in Australia. *Plant Dis.* 80:174-178.
- Gundersen, D. E., Lee, I. M., Rehner, S. A., Davis, R. E., and Kingsbury, D. T. 1994. Phylogeny of mycoplasma-like organisms (phytoplasmas): A basis for their classification. *J. Bacteriol.* 176:5244-5254.
- IRPCM Phytoplasma/Spiroplasma Working Team, P. T. G. 2004. ‘*Candidatus* Phytoplasma’, a taxon for the wall-less, non-helical prokaryotes that colonize plant phloem and insects. *Int. J. Syst. Evol. Microbiol.* 54:1243-1255.
- Itoh, T., Martin, W., and Nei, M. 2002. Acceleration of genomic evolution caused by enhanced mutation rate in endocellular symbionts. *Proc. Natl. Acad. Sci. USA* 99(20):12944-12948.
- Lathe, W. C., and Bork, P. 2001. Evolution of *tuf* genes: Ancient duplication, differential loss and gene conversion. *FEBS Lett.* 502:113-116.
- Lee, I. M., Davis, R. E., and Gundersen-Rindal, D. E. 2000. Phytoplasma: Phytopathogenic Mollicutes. *Annu. Rev. Microbiol.* 54:221-255.
- Lee, I.-M., Gundersen-Rindal, D. E., Davis, R. E., Bottner, K. D., Marcone, C., and Seemüller, E. 2004. ‘*Candidatus* Phytoplasma asteris’, a novel phytoplasma taxon associated with aster yellows and related diseases. *Int. J. Syst. Evol. Microbiol.* 54:1037-1048.
- Lee, I. M., Hammond, R. W., Davis, R. E., and Gundersen, D. E. 1993. Universal amplification and analysis of pathogen 16S rDNA for classification and identification of mycoplasma-like organisms. *Phytopathology* 83:834-842.
- Liefting, L. W., Andersen, M. T., Beever, R. E., Gardner, R. C., and Forster, R. L. S. 1996. Sequence heterogeneity in the two 16S rRNA genes of *Phormium* yellow leaf phytoplasma. *Appl. Environ. Microbiol.* 62:3133-3139.
- Liefting, L. W., Beever, R. E., Winks, C. J., Pearson, M. N., and Forster, R. L. S. 1997. Planthopper transmission of *Phormium* yellow leaf phytoplasma. *Australas. Plant Pathol.* 26:148-154.
- Liefting, L. W., Padovan, A. C., Gibb, K. S., Beever, R. E., Andersen, M. T., Newcomb, R. D., Beck, D. L., and Forster, R. L. S. 1998. ‘*Candidatus* Phytoplasma australiense’ is the phytoplasma associated with Australian grapevine yellows, papaya dieback and *Phormium* yellow leaf diseases. *Eur. J. Plant Pathol.* 104:619-623.
- Lim, P.-O., and Sears, B. B. 1989. 16S rRNA sequence indicates that plant-pathogenic mycoplasma-like organisms are evolutionarily distinct from animal mycoplasmas. *J. Bacteriol.* 171:5901-5906.
- Magarey, P. A., Plavsic, B., and Wachtel, M. F. 1988. MLO associated with Australian grapevine yellows diseased phloem cells. *Int. J. Trop. Plant Dis.* 6:175-179.
- Maniloff, J. 2002. Phylogeny and evolution. Pages 31-43 in: *Molecular Biology and Pathogenicity of Mycoplasmas*. S. Razin and R. Herrmann, eds. Kluwer Academic/Plenum, New York.
- McCoy, R. E., Caudwell, A., Chang, C. J., Chen, T.-A., Chiykowski, L. N., Cousin, M. T., Dale, J. L., de Leeuw, G. T. N., Golino, D. A., Hackett, K. J., Kirkpatrick, B. C., Marwitz, R., Petzold, H., Shina, R. H., Sugiura, M., Whitcomb, R. F., Yang, I. L., Zhu, B. M., and Seemüller, E. 1989. Plant diseases associated with mycoplasma-like organisms. Pages 545-560 in: *The Mycoplasmas*, vol. 5. R. F. Whitcomb and J. G. Tully, eds. Academic Press, New York.
- Murray, R. G. E., and Schleifer, K. H. 1994. Taxonomic notes: A proposal for recording the properties of putative taxa of prokaryotes. *Int. J. Syst. Bacteriol.* 44:174-176.
- Nei, M., and Gojobori, T. 1986. Simple methods for estimating the numbers of synonymous and nonsynonymous nucleotide substitutions. *Mol. Biol. Evol.* 3:418-426.
- Padovan, A., Gibb, K., and Persley, D. 2000. Association of ‘*Candidatus* Phytoplasma australiense’ with green petal and lethal yellows diseases in strawberry. *Plant Pathol.* 49:362-369.
- Parker, M. A., Doyle, J. L., and Doyle, J. J. 2004. Comparative phylogeography of *Amphicarpea* legumes and their root-nodule symbionts in Japan and North America. *J. Biogeogr.* 31:425-434.
- Rozas, J., Sanchez-DelBarrio, J., Messegue, X., and Rozas, R. 2003. DnaSP, DNA polymorphism analyses by the coalescent and other methods. *Bioinformatics* 19:2496-2497.

35. Sanmartin, I., and Ronquist, F. 2004. Southern hemisphere biogeography inferred by event-based models: Plant versus animal patterns. *Syst. Biol.* 53:216-243.
36. Schneider, B., Gibb, K. S., and Seemüller, E. 1997. Sequence and RFLP analysis of the elongation factor Tu gene used in differentiation and classification of phytoplasmas. *Microbiology* 143:3381-3389.
37. Schneider, B., Padovan, A., De La Rue, S., Eichner, R., Davis, R., Bernuetz, A., and Gibb, K. 1999. Detection and differentiation of phytoplasmas in Australia: An update. *Aust. J. Agric. Res.* 50:333-342.
38. Schneider, B., and Seemüller, E. 1994. Presence of two sets of ribosomal genes in phytopathogenic Mollicutes. *Appl. Environ. Microbiol.* 60:3409-3412.
39. Schneider, B., Seemüller, E., Smart, C. D., and Kirkpatrick, B. C. 1995. Phylogenetic classification of plant pathogenic mycoplasmalike organisms or phytoplasmas. Pages 369-380 in: *Molecular and Diagnostic Procedures in Mycoplasmaology*. I. R. Razin and J. G. Tully, eds. Academic Press, San Diego, CA.
40. Seemüller, E., Garnier, M., and Schneider, B. 2002. Mycoplasmas of plant and insects. Pages 91-122 in: *Molecular Biology and Pathogenicity of Mycoplasmas*. S. Razin and R. Herrmann, eds. Kluwer Academic/Plenum, New York.
41. Seemüller, E., Marcone, C., Lauer, U., Ragozzino, A., and Göschl, M. 1998. Current status of molecular classification of the phytoplasmas. *J. Plant Pathol.* 80:3-26.
42. Smart, C. D., Schneider, B., Blomquist, C. L., Guerra, L. J., Harrison, N. A., Ahrens, U., Lorenz, K. H., Seemüller, E., and Kirkpatrick, B. C. 1996. Phytoplasma-specific PCR primers based on sequences of the 16S-23S rRNA spacer region. *Appl. Environ. Microbiol.* 62:2988-2993.
43. Streten, C., and Gibb, K. 2005. Genetic variation in '*Candidatus* Phytoplasma australiense'. *Plant Pathol.* 54:8-14.
44. Streten, C., Herrington, M. E., Hutton, D. G., Persley, D. M., Waite, G. K., and Gibb, K. S. 2005 Plant hosts of the phytoplasmas and rickettsia-like-organisms associated with strawberry lethal yellows and green petal diseases. *Australas. Plant Pathol.* 34:165-173.
45. Swofford, D. 1998. *PAUP* Phylogenetic Analysis Using Parsimony and Other Methods*. Sinauer, Sunderland, MA.
46. Thompson, J. D., Gibson, T. J., Plewniak, F., Jeanmougin, F., and Higgins, D. G. 1997. The CLUSTAL_X windows interface: Flexible strategies for multiple sequence alignment aided by quality analysis tools. *Nucleic Acids Res.* 25:4876-4882.
47. Wernegreen, J. J. 2002. Genome evolution in bacterial endosymbionts of insects. *Nat. Rev. Genet.* 3:850-861.
48. White, D. T., Billington, S. J., Walsh, K. B., and Scott, P. T. 1997. DNA sequence analysis supports the association of phytoplasmas with papaya (*Carica papaya*) dieback, yellow crinkle and mosaic. *Australas. Plant Pathol.* 26:28-36.
49. Wood, G. A. 1998. *Virus and Virus-Like Diseases and Non-infectious Disorders of Small Fruits in New Zealand*. Royal Society New Zealand, Wellington.