

'*Candidatus Liberibacter solanacearum*', associated with plants in the family Solanaceae

Lia W. Liefiting,^{1†} Bevan S. Weir,^{2†} Shaun R. Pennycook² and Gerard R. G. Clover¹

Correspondence

Lia W. Liefiting
lia.liefiting@maf.govt.nz

¹Plant Health and Environment Laboratory, MAF Biosecurity New Zealand, PO Box 2095, Auckland 1140, New Zealand

²Landcare Research, Private Bag 92170, Auckland Mail Centre, Auckland 1142, New Zealand

A liberibacter (isolate NZ082226) was detected in a symptomatic tomato plant and subsequently in five other members of the family Solanaceae: capsicum, potato, tamarillo, cape gooseberry and chilli. Phylogenetic analyses of the 16S rRNA gene sequence, the deduced amino acid sequence of the *rplJ* gene and a partial nucleotide sequence of the β operon indicated that isolate NZ082226 represents a novel candidate species of '*Candidatus Liberibacter*', for which the name '*Candidatus Liberibacter solanacearum*' is proposed.

The candidate genus '*Candidatus Liberibacter*' was first proposed by Jagoueix *et al.* (1994) with two members, '*Candidatus Liberibacter asiaticum*' and '*Candidatus Liberibacter africanum*'. Later, they were renamed '*Candidatus Liberibacter asiaticus*' and '*Candidatus Liberibacter africanus*' in order to conform with the International Code of Nomenclature of Bacteria (Garnier *et al.*, 2000). In 2004, a third candidate species, '*Candidatus Liberibacter americanus*', was described, from Brazil (Teixeira *et al.*, 2005). Collectively, these three liberibacter species are commonly known as huanglongbing (formerly citrus greening). They have only been found naturally to infect citrus and other members of the family Rutaceae.

The liberibacters are phloem-limited, Gram-negative, unculturable bacteria that are spread from infected to healthy plants by grafting and psyllid insect vectors (reviewed by Bové, 2006). Scanning electron microscopy images of liberibacters reveal that they have a rod-shaped morphology (Tanaka *et al.*, 2007). '*Candidatus Liberibacter*' species belong to the *Alphaproteobacteria*, with their closest relatives belonging to the genera *Bradyrhizobium*, *Bartonella*, *Agrobacterium*, *Brucella* and *Afipia* (Jagoueix *et al.*, 1994).

An investigation to determine the aetiology of a new disease of glasshouse-grown tomato (*Solanum lycopersicum*) and capsicum (*Capsicum annuum*) in New Zealand was done by Liefiting *et al.* (2009). Extensive testing ruled out the presence of pathogenic fungi, culturable bacteria, viruses, viroids and phytoplasmas. Transmission electron microscopy of thin

sections of leaf tissue from symptomatic plants revealed the presence of phloem-limited bacterium-like organisms. A range of universal and specific 16S rRNA PCR primers were used in different combinations on DNA extracted from healthy and symptomatic plants. One of the primer combinations produced a unique product from symptomatic plants only. Sequence analysis of this fragment revealed that it shared high identity with '*Candidatus Liberibacter*' species. The remainder of the 16S rRNA gene was sequenced and phylogenetic analysis showed that it is distinct from the three citrus-infecting liberibacter species described previously (Liefiting *et al.*, 2009). Subsequently, with the development of a specific PCR diagnostic method, this new liberibacter was also detected in potato (*Solanum tuberosum*), tamarillo (*Solanum betaceum*), cape gooseberry (*Physalis peruviana*) (Liefiting *et al.*, 2008a, b; Abad *et al.*, 2009) and chilli (*Capsicum* sp.).

A 1.7 kb fragment of the β operon containing the ribosomal proteins L10 (*rplJ*) and L12 (*rplL*) and the RNA polymerase beta subunit (*rpoB*) was amplified using the primer pair fp 1898/rp 1897 (Planet *et al.*, 1995). From the resulting sequence, the primers rp01F (5'-CTCT-AAGATTTTCGGTTGGTT-3') and rp01R (5'-TATATCT-ATCGTTGCACCAG-3') were designed in order to obtain the full-length sequence of the 1.7 kb PCR product. PCR products were sequenced directly and overlapping fragments were assembled using the SeqMan software of the LaserGene package. Multiple sequence alignments were performed using Prankster (Löytynoja & Goldman, 2008) including sequences from other liberibacters and the genome sequences of related taxa. Multiple methods of phylogenetic tree building were used; all were generally congruent. Bayesian inference trees are presented here as representative. Trees were constructed with MrBayes 3.12 (Huelsenbeck & Ronquist, 2001; Ronquist & Huelsenbeck,

†These authors contributed equally to this work.

The GenBank/EMBL/DDBJ accession numbers for the 16S rRNA gene sequence and the 16S–23S rRNA intergenic spacer and for a fragment of the β operon of '*Candidatus Liberibacter solanacearum*' isolate NZ082226 are EU834130 and EU834131, respectively.

2003) using the GTR + I + Γ model for DNA and the mixed model for amino acid sequences. Analyses were run for 5×10^6 generations using a flat Dirichlet distribution (assuming no prior knowledge on taxa relations). The first 2×10^6 generations before convergence of likelihoods were discarded from the analysis.

Phylogenetic analyses based on sequences of the 16S rRNA gene (Liefting *et al.*, 2009), the deduced RplJ protein (Fig. 1) and the β operon (Fig. 2) show that isolate NZ082226 is unambiguously a member of ‘Candidatus Liberibacter’ and is distinct from the currently described candidate species and strains. The exact phylogenetic relationship to the other liberibacters is uncertain. In the tree based on β operon nucleotide sequences (Fig. 2), isolate NZ082226 was located at the base with ‘Ca. L. americanus’; however, in the *rplJ* deduced amino acid sequence tree (Fig. 1), it was located between ‘Ca. L. asiaticus’ and ‘Ca. L. africanus’. The posterior probability for this node is also low (0.55), indicating uncertainty. The sequence divergence of isolate NZ082226 may be the cause of this uncertainty, as a result of long-branch attraction (Bergsten, 2005). Pairwise nucleotide identity matrices of the *rpoB* sequence were performed using MatGat (Campanella *et al.*, 2003). Isolate NZ082226 had 88.1% identity to ‘Ca. L. asiaticus’, 89.1% to ‘Ca. L. africanus’, 87.4% to ‘Ca. L. africanus subsp. capensis’ and 82.5% to ‘Ca. L. americanus’. Under the proposal of Adékambi *et al.* (2008), members of the same species have an *rpoB* gene sequence identity of $>97.7\%$ and members of different genera show identity of $<85.5\%$. With these criteria, isolate NZ082226 represents a novel species in the same genus as ‘Ca. L. asiaticus’, ‘Ca. L. africanus’ and ‘Ca. L. africanus subsp. capensis’. However, this proposal would determine ‘Ca. L. americanus’ to be in a separate genus and, with an identity score of 92.1%, ‘Ca. L. africanus’ and ‘Ca. L.

africanus subsp. capensis’ would be considered different species.

The 16S rRNA gene and approximately 580 bp of the β operon including partial *rplJ* and *rplL* genes were sequenced from the liberibacters isolated from capsicum, potato, tamarillo, cape gooseberry and chilli. All sequences were identical to that of isolate NZ082226 from tomato.

Naming of the citrus-infecting liberibacter species is based on the continent where they were first detected and occurred. We propose that the novel liberibacter species identified in solanaceous plants is named after its plant host family rather than the geographical region. Although this liberibacter species was first identified in New Zealand, it has subsequently been detected in potato plants exhibiting zebra chip symptoms in the USA (Abad *et al.*, 2009). Zebra chip disease of potato was first reported in Mexico in 1994 and then in the USA in 2000 (Secor & Rivera-Varas, 2004). Transmission studies by Munyaneza *et al.* (2007) strongly suggest that the tomato/potato psyllid *Bactericera cockerelli* is the vector of the zebra chip pathogen. *B. cockerelli* has a very wide host range, including species in 20 plant families, with a strong preference for solanaceous species (Wallis, 1955). Therefore, isolate NZ082226 could potentially infect a wider host range than the six solanaceous hosts identified to date.

Evidence suggests that the solanaceae-infecting liberibacter did not originate in New Zealand but came into the country with the psyllid vector. *B. cockerelli* was first discovered in an Auckland glasshouse tomato crop in May 2006, after which the plant disease symptoms caused by the liberibacter were first observed. Furthermore, the liberibacter has been detected by PCR in individuals from a colony of *B. cockerelli* started from one of the initial

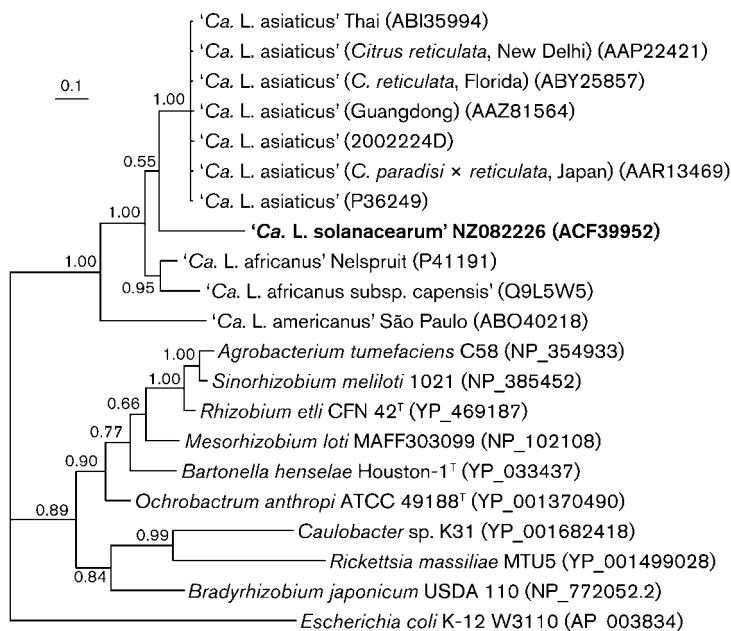


Fig. 1. Fifty-per-cent majority-rule consensus Bayesian inference phylogenetic tree based on *rplJ* deduced amino acid sequences showing the relationships of the four ‘Candidatus Liberibacter’ species and representatives of the Alphaproteobacteria. The sequence of *Escherichia coli* K-12 W3110 was used as an outgroup. GenBank accession numbers are indicated. Posterior probabilities appear at nodes. Bar, 0.1 inferred changes per site.

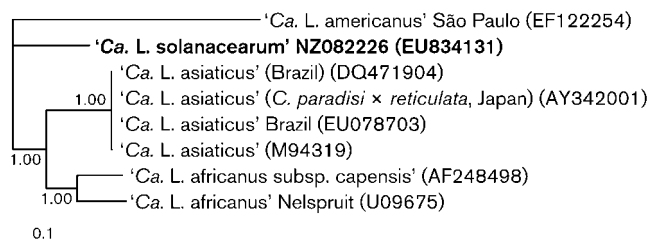


Fig. 2. Unrooted Bayesian inference phylogenetic tree based on β operon nucleotide sequences showing the relationships of the four 'Candidatus Liberibacter' species. Posterior probabilities appear at nodes. GenBank accession numbers are indicated. Bar, 0.1 inferred changes per site.

incursion sites [Peter Workman (New Zealand Institute for Crop & Food Research Ltd), personal communication].

The results of phylogenetic analyses obtained in the present and previous studies, along with the novel host-range, show that isolate NZ082226 represents a novel candidate species within the genus 'Candidatus Liberibacter', for which the name 'Candidatus Liberibacter solanacearum' is proposed. This is the first liberibacter species described from a non-rutaceous host.

Description of 'Candidatus Liberibacter solanacearum'

'Candidatus Liberibacter solanacearum' (so.la.na.ce.a'rum. N.L. fem. pl. n. *Solanaceae* a botanical family; N.L. gen. pl. n. *solanacearum* of the Solanaceae, referring to the family of plant hosts, capsicum, tomato, potato, tamarillo, cape gooseberry and chilli, from which the bacterium has been isolated).

Reference isolate is NZ082226, isolated from a tomato plant in South Auckland, New Zealand. DNA and freeze-dried plant material from this isolate are available from the authors.

[(*Alphaproteobacteria*) NC; G-; R; NAS (GenBank accession numbers EU834130 for the 16S rRNA gene and 16S–23S rRNA intergenic spacer and EU834131 for the β operon), oligonucleotide sequence of unique region of 16S rRNA 5'-GCGCTTATTTTAAATAGGAGCGGCA-3'; S (*Capsicum annuum*, *Physalis peruviana*, *Solanum betaceum*, *Solanum lycopersicum*, *Solanum tuberosum*, phloem; *Bactericera cockerelli* (Psyllidae), tissue unknown; M].

Acknowledgements

We thank Drs Peter Johnston and Ross Beever from Landcare Research for valuable discussions.

References

Abad, J. A., Bandla, M., French-Monar, R. D., Liefing, L. W. & Clover, G. R. G. (2009). First report of the detection of 'Candidatus Liberibacter' species in zebra chip disease-infected potato plants in the United States. *Plant Dis* **93**, 108.

Adékambi, T., Shinnick, T. M., Raoult, D. & Drancourt, M. (2008). Complete *rpoB* gene sequencing as a suitable supplement to DNA–DNA hybridization for bacterial species and genus delineation. *Int J Syst Evol Microbiol* **58**, 1807–1814.

Bergsten, J. (2005). A review of long-branch attraction. *Cladistics* **21**, 163–193.

Bové, J. M. (2006). Huanglongbing: a destructive, newly-emerging, century-old disease of citrus. *J Plant Pathol* **88**, 7–37.

Campanella, J. J., Bitincka, L. & Smalley, J. (2003). MatGAT: an application that generates similarity/identity matrices using protein or DNA sequences. *BMC Bioinformatics* **4**, 29.

Garnier, M., Jagoueix-Eveillard, S., Cronje, P. R., Le Roux, H. F. & Bové, J. M. (2000). Genomic characterization of a liberibacter present in an ornamental rutaceous tree, *Calodendrum capense*, in the Western Cape province of South Africa. Proposal of 'Candidatus Liberibacter africanus subsp. capensis'. *Int J Syst Evol Microbiol* **50**, 2119–2125.

Huelsenbeck, J. P. & Ronquist, F. (2001). MrBayes: Bayesian inference of phylogenetic trees. *Bioinformatics* **17**, 754–755.

Jagoueix, S., Bové, J. M. & Garnier, M. (1994). The phloem-limited bacterium of greening disease of citrus is a member of the α subdivision of the Proteobacteria. *Int J Syst Bacteriol* **44**, 379–386.

Liefing, L. W., Perez-Egusquiza, Z. C., Clover, G. R. G. & Anderson, J. A. D. (2008a). A new 'Candidatus Liberibacter' species in *Solanum tuberosum* in New Zealand. *Plant Dis* **92**, 1474.

Liefing, L. W., Ward, L. I., Shiller, J. B. & Clover, G. R. G. (2008b). A new 'Candidatus Liberibacter' species in *Solanum betaceum* (tamarillo) and *Physalis peruviana* (cape gooseberry) in New Zealand. *Plant Dis* **92**, 1588.

Liefing, L. W., Sutherland, P. W., Ward, L. I., Paice, K. L., Weir, B. S. & Clover, G. R. G. (2009). A new 'Candidatus Liberibacter' species associated with diseases of solanaceous crops. *Plant Dis* **93**, 208–214.

Löytynoja, A. & Goldman, N. (2008). Phylogeny-aware gap placement prevents errors in sequence alignment and evolutionary analysis. *Science* **320**, 1632–1635.

Munyaneza, J. E., Crosslin, J. M. & Upton, J. E. (2007). Association of *Bactericera cockerelli* (Homoptera: Psyllidae) with "zebra chip," a new potato disease in Southwestern United States and Mexico. *J Econ Entomol* **100**, 656–663.

Planet, P., Jagoueix, S., Bové, J. M. & Garnier, M. (1995). Detection and characterization of the African citrus greening Liberobacter by amplification, cloning, and sequencing of the *rplKALJL-rpoBC* operon. *Curr Microbiol* **30**, 137–141.

Ronquist, F. & Huelsenbeck, J. P. (2003). MrBayes 3: Bayesian phylogenetic inference under mixed models. *Bioinformatics* **19**, 1572–1574.

Secor, G. A. & Rivera-Varas, V. V. (2004). Emerging diseases of cultivated potato and their impact on Latin America. *Rev Latinoam Papa* **1** (Suppl.), 1–8.

Tanaka, F. A. O., Colleta-Filho, H. D., Alves, K. C. S., Spinelli, M. O., Machado, M. A. & Kitajima, E. W. (2007). Detection of the 'Candidatus Liberibacter americanus' in phloem vessels of experimentally infected *Catharanthus roseus* by scanning electron microscopy. *Fitopatol Bras* **36**, 519–520.

Teixeira, D. C., Saillard, C., Eveillard, S., Danet, J. L., Inácio da Costa, P., Ayres, A. J. & Bové, J. (2005). 'Candidatus Liberibacter americanus', associated with citrus huanglongbing (greening disease) in São Paulo State, Brazil. *Int J Syst Evol Microbiol* **55**, 1857–1862.

Wallis, R. L. (1955). *Ecological studies on the potato psyllid as a pest of potatoes*. USDA Technical Bulletin 1107. Washington, DC: US Department of Agriculture.