Citrus R&T (e-ISSN 2236-3122)
http://citrusrt.centrodecitricultura.br
doi:10.5935/2236-3122.20120009

Comunicação/Note

Reported single nucleotide polymorphisms on the 16S rRNA gene do not support haplotypes of "*Candidatus* Liberibacter asiaticus"

Warrick R. Nelson[1*]

SUMMARY

"*Candidatus* Liberibacter asiaticus" (Las) causes huanglongbing, also known as citrus greening, in citrus crops widespread across the major citrus production areas of the world, especially Asia, Brazil and southern USA. Some studies suggest that single nucleotide polymorphisms on the 16S rRNA gene represent haplotypes and that these represent strains within geographic regions. Re-analysis of all available accessions of the rDNA sequences deposited in GenBank and identified as Las fails to support the reported presence of unique strains based on this taxonomically important rDNA sequence. Therefore geographically described strains also fail.

Index terms: alpha-proteobacteria, geographic variation, haplotype, huanglongbing.

RESUMO

Análises de polimorfismos de SNPs no gene 16S rRNA não confirmam haplótipos de "*Candidatus* Liberibacter asiaticus"

"Candidatus Liberibacter asiaticus" (Las) causa o *huanglongbing*, também conhecido como *greening*, em pomares localizados em áreas de produção de citros importantes do mundo, especialmente na Ásia, Brasil e sul dos EUA. Alguns estudos sugerem que polimorfismos de nucleotídeos no gene 16S rRNA representam haplótipos, sendo estes representados por estirpes dentro de regiões geográficas. Uma nova análise de todos os acessos disponíveis das sequências de rDNA depositadas no GenBank e identificadas como Las não confirmou a presença de estirpes únicas relacionadas com as regiões geográficas.

Termos de indexação: alpha-proteobacteria, variação geográfica, haplótipo, *huanglongbing*.

[1] The New Zealand Institute for Plant & Food Research Ltd, Private Bag 4704, Christchurch 8140, New Zealand
[*] Autor para correspondência - e-mail: Warrick.Nelson@plantandfood.co.nz

COMMUNICATION

"*Candidatus* Liberibacter asiaticus" (Las) causes the serious citrus disease huanglongbing (HLB), also known as citrus greening (Jagoueix *et al.*, 1994). Diversity based on single nucleotide polymorphisms (SNPs) on the 16S rRNA gene has been reported (Adkar-Purushothama *et al.,* 2009; Miyata *et al.*, 2011). However, several other studies have failed to confirm diversity on this gene Khairulmazmi *et al.*, 2009; Subandiyah *et al.*, 2000; Tomimura *et al.*, 2009). Of possible significance is that the first two reports claimed SNP variation along geographic lines in two different regions of Asia, while the other studies dealt largely only with sequences derived from American isolates. In the related bacterium "*Ca*. Liberibacter solanacearum" (Lso) four haplotypes have been differentiated by SNP analysis of the 16S rRNA gene and which also indicate both geographic and host organism differences (Nelson *et al.*, 2011, 2012).

The 16S rRNA gene is taxonomically significant and is specifically targeted for phylogenetic studies, especially for fastidious bacteria (Weisburg *et al.*, 1991). Species of "*Candidatus* Liberibacter" have yet to be cultured, hence the Candidatus status of this genus. Variation on this gene can be useful to determine differentiation between different geographic or host organism sources.

This study was conducted as a re-analysis of all Las 16S rDNA sequences deposited in GenBank to determine whether the discrepancy in reports of 16S variation can be resolved and secondly whether this variation has a geographic origin using geographic designations where available on the metadata associated with the deposited sequences. Comparison of sequences across all available sequence accessions should reveal random errors in the sequences, regardless of how these errors arose.

The NCBI database (GenBank) was interrogated as at 19 December 2011 using the search term "*Candidatus* Liberibacter asiaticus"[Organism] AND 16S[All Fields] NOT psy62[All Fields], resulting in 245 hits, which were downloaded in FASTA format. A few accessions were not actually within the 16S gene region and were removed, as well as accessions less than 200 bp long. The resulting 175 accessions were aligned in ClustalX (AY919311 and AY192576 were aligned as the reverse complement). The accessions and geographic designations indicated in the metadata are listed in Table 1.

Following alignment of the 175 sequences, a 302 bp segment common to all these sequences was used to assess SNPs numbers and distribution across the accessions. This segment corresponds to bp 417290-417592, 786982-787284 and 855022-855324 on the reference genome sequence CP001677.4 (Duan *et al*., 2009). The three copies of the 16S rDNA gene in this genome sequence were also compared. Four insertions in the reverse complement version (416812-418322) distinguish this version from the other two copies. However, these insertions did not occur in the 302 bp fragment, indicating that none of the SNPs between the three copies in the genome will be present within this analysis.

These 16S rDNA sequences showed 118 accessions being identical and the remaining 47 exhibited 73 SNPs (supplementary FASTA file). Thirty-one of these accessions had more than one SNP (Table 1). Of these 73 SNPs, most occurred on a single accession, suggesting inconsistencies in sequencing, including some reporting "N" or "R". A few SNPs occurred on more than one accession (Table 2), suggesting strains as reported earlier (Adkar-Purushothama *et al.,* 2009; Miyata *et al.*, 2011). However, this was not a clear indication of strains, as accessions sharing a SNP in one position seldom shared the same SNP elsewhere along the sequence. FJ263698 and FJ263702 are the only two sequences sharing more than a single SNP (SNP 1 and 23 in Table 2), no other sequences had more than one common SNP (although a number shared designation "N" in the same position).

The result of this re-analysis does not provide confidence to confirm haplotypes of Las based on the16S rDNA sequences. Only 27% of sequences showed SNPs in the segment studied and these suggested misreads in the sequencing rather than genuine haplotypes. Even aligning the full available sequence for each accession simply indicated a range of SNPs in the sequences beyond the 302 bp segment analysed here, with no patterns in SNP positions or specific base changes. The advantage of a re-analysis approach is that sequences from a wide range of studies and laboratories can be compared at one time.

Table 1. Accession numbers and the geographic metadata of "*Candidatus* Liberibacter asiaticus" (Las)16S rDNA sequences from the GenBank database. Thirty-one accessions (marked *) have more than one single nucleotide polymorphism (SNP) in the 302 bp sequence

Geographic Region	Accession
Belize	GQ502291.1; GU061003.1
Brazil	AY919311.1; DQ471901.1; *EU921613.1; EU921622.1
China	AY192576.1; DQ157273.1; DQ157274.1; *DQ157275.1; *DQ303210.1; DQ431997.1; DQ431998.1; DQ431999.1; *DQ432000.1; *DQ432001.1; DQ432002.1; *DQ432003.1; DQ432004.1; DQ432005.1; *DQ778016.1; EU921614.1; EU921615.1; EU921616.1; *EU999026.1; *EU999027.1; JF731334.1; JF731335.1; JF731336.1; JF731337.1; JF731338.1; JF731339.1; JF731340.1; JF731341.1; JN211030.1; JN211031.1; JN211032.1; JN211033.1; JN211035.1; JN211036.1; JN211037.1
Cuba	FJ544941.1
Dominican Republic	FJ811891.1; FJ811892.1; FJ811893.1; FJ811894.1; FJ811895.1; FJ821709.1; FJ821710.1; FJ821711.1; FJ821712.1; FJ821713.1; FJ821714.1; FJ821715.1; FJ821716.1; FJ821717.1; FJ821718.1
East Timor	AB555706.1
Florida	DQ471900.1; EU130552.1; EU130553.1; EU130554.1; EU130555.1; EU265646.1; *EU921617.1; EU921618.1; FJ236554.1; FJ263696.1; FJ263697.1; *FJ263698.1; *FJ263699.1; *FJ263700.1; *FJ263701.1; *FJ263702.1; FJ263703.1; *FJ263704.1; *FJ750456.1; FJ750457.1
India	AB555708.1; AB558579.1; AB558580.1; EF552698.1; EF552699.1; EU939452.1; FJ196314.1; FJ765088.1; FJ827777.1; FJ827779.1; *GQ369792.1; *L22532.1
Indonesia	AB480087.1; AB480088.1; AB480089.1; AB480090.1; AB480091.1; AB480092.1; AB480093.1; AB480094.1; AB480095.1; AB480096.1; AB480097.1; AB480098.1; AB480099.1; AB480100.1; AB480101.1; AB480102.1
Iran	HQ335313.1; JN049632.1; JN049636.1
Jamaica	JN245973.1; *JN245974.1; *JN245975.1; JN245976.1; JN245977.1; JN245980.1; JN245981.1
Japan	AB480072.1; AB480073.1; AB480074.1; AB480075.1
Louisiana	*FJ750458.1; *FJ750459.1
Malaysia	EU224393.1; EU224394.1; EU371106.1; GU133055.1
Papua New Guinea	AB555707.1
Puerto Rico	GU139343.1; GU139344.1; GU139345.1
Taiwan	AB480076.1; AB480077.1; AB480078.1; AB480079.1; AB480080.1; DQ302750.1
Thailand	AB480086.1
unspecified	EU130556.1; GQ254604.1; GQ254605.1; GQ254606.1; *GQ254607.1; GQ254608.1; GQ254609.1; GQ254610.1; GQ254611.1; *GQ254612.1; GQ254613.1; GQ254614.1; GQ254615.1; GQ254616.1; GQ254617.1; GQ254618.1; GQ254619.1; *GQ254620.1; GQ254621.1; GQ254622.1; GQ254623.1; GQ254624.1; GQ254625.1; GQ254626.1; GQ254627.1; *GQ254629.1; *GQ254630.1; *GQ254631.1; GQ254632.1; GQ254633.1; *GU991649.1; GU991650.1; GU991651.1; *GU991652.1
Vietnam	AB480081.1; AB480082.1; AB480083.1; AB480084.1; AB480085.1

Table 2. "*Candidatus* Liberibacter asiaticus" (Las) 16S rDNA sequences where more than one accession showed a single nucleotide polymorphism (SNP) at the same position and some positions had more than one SNP

SNP #	SNP	Sequences
1	T/C	FJ263698; FJ263702
3	C/T	FJ750456; FJ750457
4	-/G	GQ254620; GQ254630; GQ254631
11	T/C	GQ254607; GQ254629
14	T/C	EU999027; EU999026; L22532
16	C/N, C/G	EU999027; EU999026; L22532
17	G/N, G/C, G/T	EU999027; EU999026; L22532; GQ254607
22	G/N	EU999027; EU999026; L22532; FJ750456
23	C/T	DQ778016; FJ263698; FJ263702
24	T/G	DQ778016; FJ263702; FJ263698
33	C/N, C/T	DQ432000; EU999027; EU999026
36	A/G	FJ263700; FJ263701
47	T/N, T/A	DQ432003; EU999027; EU999026; GQ369792
48	A/G, A/T	GQ254607; GQ254612
49	T/C	DQ303210; DQ157275
50	A/-	FJ263699; FJ263704
51	-/C	FJ750458; FJ750459

A lack of genetic diversity suggests a commonality of source of this bacterium from its origin into all the current geographic regions. This spread has been suggested as being largely anthropogenic, although the ultimate geographic origin is disputed (Beattie *et al.*, 2008). Conservation of genes is likely to be particularly high in this species considering it has a lifestyle requiring propagation in both plant and insect host stages. Other genetic regions might be more susceptible to genetic variation, such as the ψserA-trmU-tufB-secE-nusGrplKAJL-rpoB gene cluster (Furuya *et al.*, 2010), the *omp* gene (Bastianel *et al.,* 2005; Miyata *et al.*, 2011), or prophage variation (Liu *et al.*, 2011). Analysis of simple sequence repeats (SSR) suggests a high degree of diversity (Katoh *et al.*, 2011). These hypervariable regions, commonly associated with virulence variation yet also potentially functionally neutral (van Belkum *et al.*, 1998), could point to much more recent genetic variation and therefore be of interest for biosecurity studies. Unfortunately, few sequences of these other regions have been deposited in GenBank and are essentially derived from a single study in each case.

This study, using 175 sequences of 16S rDNA deposited in GenBank, failed to confirm SNP patterns and therefore potential haplotypes. This contrasts strongly with a similar analysis of Lso where sequences indicated three haplotypes. Until further sequences are available and indicate otherwise, Las should be considered to be a single strain in all geographic regions.

REFERENCES

Adkar-Purushothama C, Quaglino F, Casati P, Ramanayaka J & Bianco P (2009) Genetic diversity among "*Candidatus Liberibacter asiaticus*" isolates based on single nucleotide polymorphisms in 16S rRNA and ribosomal protein genes. *Annals of microbiology*, 59:681-688.

Bastianel C, Garnier-Semancik M, Renaudin J, Bové J & Eveillard S (2005) Diversity of "*Candidatus Liberibacter asiaticus*" based on the omp gene sequence. *Applied and environmental microbiology*, 71:6473-6478.

Beattie G, Holford P, Mabberley D, Haigh A & Broadbent P (2008) On the origins of Citrus, Huanglongbing, *Diaphorina citri* and *Trioza erytreae*. *International research conference on Huanglongbing, Florida, USA*, 25-57.

Duan Y, Zhou L, Hall D, Li W, Doddapaneni H, Lin H, Liu L, Vahling C, Gabriel D, Williams K, Dickerman A, Sun Y & Gottwald T (2009) Complete genome sequence of citrus huanglongbing bacterium, "*Candidatus Liberibacter asiaticus*" obtained through metagenomics. *Molecular plant microbe interactions*, 22:1011-1020.

Furuya N, Matsukura K, Tomimura K, Okuda M, Miyata S & Iwanami T (2010) Sequence homogeneity of the ψserA-trmU-tufB-secE-nusG-rplKAJL-rpoB gene cluster and the flanking regions of "*Candidatus Liberibacter asiaticus*" isolates around Okinawa main island in Japan. *Journal of general plant pathology*, 76:122-131.

Jagoueix S, Bové J & Garnier M (1994) The phloem-limited bacterium of greening disease of citrus is a member of the α-subdivision of the Proteobacteria. *International journal of systematic bacteriology*, 44:379-386.

Katoh H, Subandiyah S, Tomimura K, Okuda M, Su H & Iwanami T (2011) Differentiation of "*Candidatus Liberibacter asiaticus*" isolates by variable number of tandem repeat analysis. *Applied environmental microbiology,* 77:1910-1917.

Khairulmazmi A, Kamaruzaman S, Habibuddin H, Jugah K & Syed Rastan S (2009) Characterization of "*Candidatus Liberibacter asiaticus*" isolated from *Citrus grandis* and *Citrus reticulata* based on 16S rDNA and outer membrane protein (OMP) gene. *International journal of agriculture and biology*, 11:401-407.

Liu R, Zhang P, Pu X, Xing X, Chen J & Deng X (2011) Analysis of a prophage gene frequency revealed population variation of "*Candidatus Liberibacter asiaticus*" from two citrus-growing provinces in China. *Plant disease,* 95:431-435.

Miyata S, Kato H, Davis R, Smith M, Weinert M & Iwanami T (2011) Asian-common strains of "*Liberibacter asiaticus*" are distributed in Northeast India, Papua New Guinea and Timor-Leste. *Journal of general plant pathology*, 77:43-47.

Nelson W, Fisher T & Munyaneza J (2011) Haplotypes of "*Candidatus Liberibacter solanacearum*" suggest long-standing separation. *European journal of plant pathology*, 130:5-12.

Nelson W, Sengoda V, Alfaro-Fernandez A, Font M, Crosslin J, Munyaneza J (2012) A new haplotype of "*Candidatus Liberibacter solanacearum*" identified in the Mediterranean region. European journal of plant pathology DOI: 10.1007/s10658-012-0121-3.

Subandiyah S, Iwanami T, Tsuyumu S & Ieki H (2000) Comparison of 16S rDNA and 16S/23S intergenic region sequences among citrus greening organisms in Asia. *Plant disease*, 84:15-18.

Tomimura K, Miyata S, Furuya N, Kubota K, Okuda M, Siti Subandiyah S, Hung T, Su H & Iwanami T, (2009) Evaluation of genetic diversity among "*Candidatus Liberibacter asiaticus*" isolates collected in Southeast Asia. *Phytopathology,* 99:1062-1069.

van Belkum A, Scherer S, van Alphen L & Verbrugh H (1998) Short-Sequence DNA Repeats in Prokaryotic Genomes. *Microbiological and moelcular biology review*, 62:275-293.

Weisburg W, Barns S, Pelletier D & Lane D (1991) 16S ribosomal DNA amplification for phylogenetic study. *Journal of bacteriology*, 173:697-703.

Recebido: 26/06/2012 – Aceito: 07/02/2013
(CRT 053-12)