Original Paper

Obtaining positive controls for the detection of two polymorphic regions in Cuban strains of '*Candidatus* Liberibacter asiaticus'

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Obtención de controles positivos para la detección de dos regiones polimórficas en aislados cubanos de '*Candidatus* Liberibacter asiaticus'

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ABSTRACT: "Huanglongbing" (HLB) is currently the most destructive disease of citrus worldwide. The detection, identification and genetic diversity studies of strains of its main associated agent '*Candidatus* Liberibacter asiaticus' were limited to the use of housekeeping genes. Because of the increasing availability of complete genome sequences of different '*Ca.* L. asiaticus' strains from different countries, regions inside the genome of these bacteria having greater variability have been successfully implemented for its characterization, including microsatellites, prophage genes and miniature inverted-repeat transposable elements (MITEs). In the present work, six Cuban strains of '*Ca*. L. asiaticus' with different geographical origins (Western, Central and Eastern regions) were included. Two polymorphic markers (prophage types and MITEs) were used to verify the presence of genetic diversity among the strains. The combination of the information obtained from detecting markers allowed verifying the strain differentiation according to the length of the amplified bands. The detected strains are important controls necessary to guarantee the performance and interpretation of tests using these molecular markers. The PCR systems used will allow a fast and improved characterization of the bacterial populations present in Cuba. This is the first report of the detection of polymorphic regions in the genome of Cuban strains of '*Ca*. L. asiaticus'.

Keywords: "Huanglongbing", polymorphic genomic regions, positive controls, Cuban strains.

RESUMEN: "Huanglongbing" (HLB) es actualmente la enfermedad más destructiva de los cítricos a nivel mundial. La detección, identificación y estudios de diversidad genética de cepas de su principal agente asociado '*Candidatus* Liberibacter asiaticus' se limitaron al empleo de genes de mantenimiento. A partir de la creciente disponibilidad de secuencias del genoma completo de diferentes cepas de '*Ca.* L. asiaticus' en varios países, regiones de mayor variabilidad, dentro del genoma de estas bacterias, se implementaron exitosamente para su caracterización, incluyendo microsatélites, los genes de origen profago y elementos transponibles miniatura con repetición invertida (MITEs por sus siglas en inglés). En el presente trabajo, se incluyeron seis aislados cubanos de '*Ca*. L. asiaticus' provenientes de diferentes orígenes geográficos (regiones Occidental, Central y Oriental). Dos marcadores polimórficos (tipos de profagos y MITEs) se utilizaron para verificar la diversidad genética entre estas cepas. La combinación de la información obtenida de la detección de ambos marcadores permitió verificar la diferenciación de las cepas acorde al tamaño de las bandas amplificadas. Las diversas cepas detectadas son controles importantes necesarios para garantizar el desarrollo e interpretación de los ensayos desarrollados utilizando estos marcadores moleculares. Los sistemas de PCR empleados permitirán una caracterización rápida y mejorada de las poblaciones de esta bacteria presentes en Cuba. Este constituye el primer informe de la detección de regiones polimórficas en el genoma de aislados cubanos de '*Ca*. L. asiaticus'.

Palabras clave: "Huanglongbing", regiones genómicas polimórficas, controles positivos, cepas cubanas.

INTRODUCTION

"Huanglongbing" (HLB), also known as citrus greening, is the most serious disease threating the citrus industry worldwide (1). Its occurrence has been associated with the presence of three bacteria within the '*Candidatus* Liberibacter' genus: '*Ca*. L. asiaticus', '*Ca*. L. africanus' and '*Ca*. L. americanus' (2). In Cuba, '*Ca*. L. asiaticus' has been the only liberibacter detected since 2007 (3, 4). The lack of cultivation of this bacterium in artificial medium encouraged the active contribution of molecular tools in most of the related research (5). Its detection, identification and genetic diversity studies have mainly been focused on the use of housekeeping genes, as well as of the 16S rRNA, the intergenic region 16S-23S (6, 7, 8), the *omp* gene (9), or the *operon β* genes (10). These approaches allowed the differentiation of the three known species (6, 7, 9, 11). However, the high conserved nature of these genes limits the discriminatory power for closely related bacteria (*i.e.*, strains of the same species) (3, 12).

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Since 2009, the availability of the complete genome of different '*Ca.* L. asiaticus' strains from different countries has increased exponentially (13, 14, 15). Subsequently, genomic regions with greater variability have been successfully implemented, including microsatellites (12, 16, 17), prophage genes (18, 19, 20), and miniature inverted-repeat transposable elements (MITEs) (21) identified inside the genome of these bacteria. In Cuba, previous strain differentiation works were based on the study of these conserved regions (3, 4). Due to their greater variability, polymorphic regions are more suitable tools to evaluate genetic variability within populations of the same species. However, to guarantee the correct amplification and interpretation of the results, it is necessary to improve these techniques by including positive controls.

In this work, the objective was to obtain positive controls for the prophages and MITEs from Cuban strains of '*Ca*. L. asiaticus'.

MATERIALS AND METHODS

Plant material and DNA extraction

Six symptomatic and one asymptomatic citrus plants maintained under greenhouse conditions were used as positive and negative amplification controls, respectively (Table 1). Total DNA was extracted from 0.6 g of midribs and petioles using a CTAB method (22). The presence/absence of '*Ca*. L. asiaticus' was verified using a PCR assay with specific primers OI1/OI2c (23).

PCR for detection of polymorphic regions

For the prophage typing analyses, the following pairs of primer were used: T1-1F/T1-1R (type 1 prophage), T2-1F/T2-1R and T2-8F/T2-8R (type 2 prophage), and 891-1F/891-1R (type 3 prophage). By the other hand, for the MITEs detection, the primer pair LapPF1-F/LapPF1-R was used. (Table 2)

Several annealing temperatures (55, 60 and 62°C) were evaluated according to the following criteria:

i. the Top Taq Master Mix (Qiagen) manufacturer recommendations,

ii. the melting temperatures, calculated with the formula

$$Tm = 2\ ℃\ (A+T) + 4\ ℃\ (C+G)$$

iii. the temperatures when the primers were designed and published.

All the amplified DNAs (5 µl) were subjected to electrophoresis on 1% agarose gel (1X Tris-acetate/EDTA), stained with ethidium bromide, and visualized under ultraviolet light in a transilluminator.

RESULTS AND DISCUSSION

For detecting prophage type 1 (T1-1F/T1-1R), the amplicon with the expected size (*i.e.*, 1,025 bp) was observed in all the positive samples when amplified with the annealing temperature of 62°C. Similar results were obtained for the prophage type 2, with the

Table 1. Cuban strains of '*Ca.* L. asiaticus' used for the detection of prophage and MITEs / Cepas cubanas de '*Ca.* L. asiaticus' utilizadas para la detección de profagos y de MITEs.

Code	Location	Citrus species	Symptoms	Type of control
LPIJ4	Isla de la Juventud, Western region	Persian lime (*Citrus latifolia* Tan.)	Blotchy mottle	Positive
A01208	Matanzas, Western region	Persian lime (*Citrus latifolia* Tan.)	Blotchy mottle	Positive
2629	Ciego de Avila, Central region	Sweet orange *(Citrus sinensis* (L.) Osb.)	Blotchy mottle	Positive
2617	Camaguey, Central region	Persian lime (*Citrus latifolia* Tan.)	Blotchy mottle	Positive
A00305	Santiago de Cuba, Eastern region	Mexican lime (*Citrus aurantifolia* (Christm.) Swing)	Blotchy mottle	Positive
A00311	Guantánamo, Eastern region	Sweet orange (*Citrus sinensis* (L.) Osb.)	Blotchy mottle	Positive
2345	IIFT greenhouse	*Citrux hystrix* DC (Swangi)	Asymptomatic	Negative

Table 2. Specific primers for the detection of types of prophage and MITEs in '*Ca.* L. asiaticus' used to type the Cuban strains / Cebadores específicos para la detección de los tipos de profagos y de MITEs de '*Ca.* L. asiaticus' utilizados para tipificar las cepas cubanas

Primers	Amplicon Type/Size (bp)	Locus target	Reference
T1-1F/T1-1R	Single/1025 bp	SC1_gp030	(24)
T2-1F/T2-1R	Single/807 bp	SC2_gp030	(24)
T2-8F/T2-8R	Single/795 bp	SC2_gp240	(24)
891-1F/891-1R	Single/950 bp	PJXGC_08	(20)
LapPF1-F/LapPF1-R	Several/B720 (720 bp), B630 (630 bp) and B350 (350bp)	CLIBASIA_05620 a CLIBASIA_05625	(21)

primer pair T2-8F/T2-8R with the same temperature for the expected amplicon of 795 bp. These amplifications were obtained with an alternative annealing temperature of 60°C. However, with the additional prophage type 2- specific primers used (T2-1F/T2-1R, with an expected amplicon of 806 bp), no amplification was obtained with any of the three annealing temperatures evaluated (Fig. 1). No amplification was observed with the detection system for prophage type 3 (primers 891-1F/891-1R) (data not shown). With the temperatures tested, none of the primers yielded PCR amplification from DNA extracted from asymptomatic (confirmed non-'*Ca*. L. asiaticus'- infected) citrus leaves.

The band with the expected size (1,025 bp) with the primers T1-1F/1R was obtained in all the positive controls with the three annealing temperatures used. Da Silva *et al*. (25) reported the same results using annealing at 55°C with this system. However, in the Cuban samples the best results were obtained with 60°C or 62°C, since a size expected-single band was amplified from all the positive samples. At lower temperature (*i.e*., 55°C), additional non-specific light bands appeared in some samples (data not shown). For prophage type 2, amplification was only detected with primers T2-8F/T2-8R, while T2-1F/T2-1R did not show bands. This observation was maintained for all the annealing temperatures used. Previous works reported that the set T2-8F/T2-8R, out of eight PCR markers for prophage type 2, amplifyed this prophage from 299 Brazilian strains of '*Ca*. L. asiaticus' evaluated (25). The results with both the Cuban and Brazilian strains highlighted the usefulness of the primers T2-8F/T2-8R. This agrees with their high specificity to prophage type 2 sequence, according to their design (24).

Based on the primers of the MITEs in the prophage region, it was possible to identify two types of amplicons, B720 and B720 + B350 (Fig. 2), by using 55°C as annealing temperature. In China, Wang et al. (21) used this detection system and reported a similar population structure in 10 samples from Guizhou. However, other genotypes were identified when the sample size was increased (21, 26).

These results allowed differentiating several strains from different geographic regions in Cuba through amplification patterns. These samples will therefore be employed as controls in future survey studies. The use of the detection of prophage types and MITEs will allow a deeper and faster characterization of the Cuban population of '*Ca*. L. asiaticus' strains circulating in the different citrus growing areas of the island. This is also the first detection of polymorphic molecular markers in the Cuban strains of '*Ca.* L. asiaticus'.

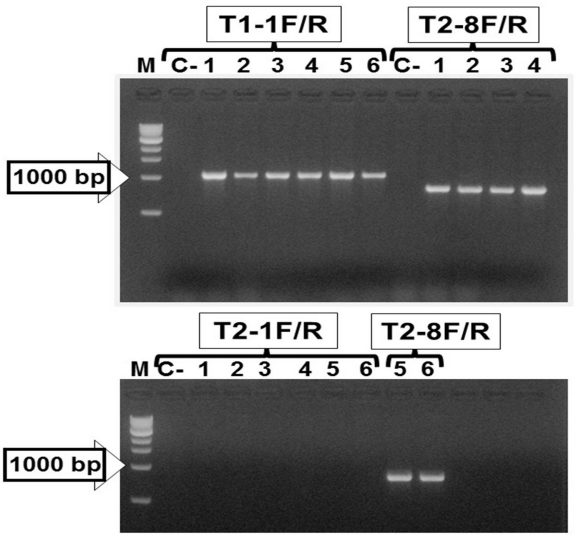

Figure 1. Detection of prophages type 1 (primers T1-1F/R), 2 (primers T2-1F/R and T2-8F/R) on control samples. Lanes 1: sample LPIJ4; 2: sample A01208; 3: sample 2629; 4: sample 2617; 5: sample A00305 y 6: sample A00311. Lanes C-: negative control 2345 and M: 1 kb DNA Ladder (BioLabs) / Detección de profagos tipo 1 (cebadores T1-1F/R), 2 (cebadores T2-1F/R y T2-8F/R) en muestras controles. Carriles 1: muestra LPIJ4; 2: muestra A01208; 3: muestra 2629; 4: muestra 2617; 5: muestra A00305 y 6: muestra A00311. Carriles C-: muestra control negativo 2345 y M: Marcador de peso molecular 1 kb (BioLabs).

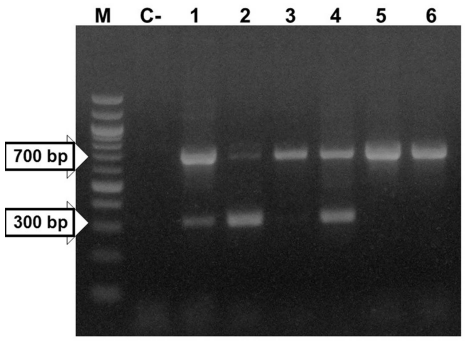

Figure 2. Profiles of bands amplified with the primers LapPF1-F/R. Lanes 1: sample LPIJ4; 2: sample A01208; 3: sample 2629; 4: sample 2617; 5: sample A00305 and 6: sample A00311. Lanes C-: negative control 2345 and M: 100 bp DNA ladder (BioLabs)/ Perfil de bandas amplificadas con los cebadores LapPF1-F/R. Carriles 1: muestra LPIJ4; 2: muestra A01208; 3: muestra 2629; 4: muestra 2617; 5: muestra A00305 y 6: muestra A00311. Carriles C-: muestra control negativo 2345 y M: Marcador de peso molecular 100 bp (BioLabs).

CONCLUSIONS

Amplification controls were obtained for the first time using diverse types of prophage and MITEs from Cuban strains of '*Ca*. L. asiaticus'. The detection of both markers is a suitable tool to evaluate the population variability of this bacterium from different geographic regions of the country.

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REFERENCES

1. Bové J-M. "Huanglongbing" or yellow shoot, a disease of Gondwanan origin: will it destroy citrus worldwide? Phytoparasitica. 2014; 42: 579-583. DOI: 20.2007/s12600-014-0415-4
2. Da Graça JV, Douhan GW, Halbert SE, Keremane ML, Lee RF, Vidalakis G. *et al.* "Huanglongbing": an overview of a complex pathosystem ravaging the world's citrus. Journal of Integrative Plant Biology. 2016; 58: 373-387. DOI: 10.1111/jjpb.12437
3. Luis M, Collazo C, Llauger R, Blanco E, Peña I, López D, *et al.* Occurrence of citrus "huanglongbing" in Cuba and association of the disease with '*Candidatus* Liberibacter asiaticus'. Journal of Plant Pathology. 2009; 91(3): 709-712. Disponible en: http://www.jstor.org/stable/41998692
4. Luis-Pantoja M, Paredes-Tomás C, Uneau Y, Myrie W, Morillon R, Satta E, *et al*. Identification of '*Candidatus* Phytoplasma' species in "huanglongbing" infected citrus orchards in the Caribbean. European Journal of Plant Pathology. 2021. DOI: 10.1007/s10658-021-02234-7
5. Deng X, Chen J, Feng Z, Shan Z, Guo H, Zhu J, et al. Identification and characterization of the "huanglongbing" bacterium in pummelo from multiple locations in Guangdong. P. R. China. Plant Disease. 2008; 92: 513-518. DOI: 10.1094/PDIS-92-4-0513
6. Jagoueix S, Bové J-M, Garnier M. The phloem-limited bacterium of greening disease of citrus is a member of the α-subdivision of the Proteobacteria. International Journal of Systematic Bacteriology. 1994; 44(3): 379-386. DOI: 10.1099/00207713-44-3-379
7. Jagoueix S, Bové J-M, Garnier M. Comparison of the 16S/23S Ribosomal Intergenic Regions of '*Candidatus* Liberobacter asiaticum' and '*Candidatus* Liberobacter africanum', the two species associated with citrus "huanglongbing" (greening) disease. International Journal of Systematic Bacteriology. 1997; 47: 224-227. DOI: 10.1099/00207713-47-1-224
8. Li W, Hartung JS, Levy L. Quantitative real-time PCR for detection and identification of '*Candidatus* Liberobacter' species associated with citrus "huanglongbing". Journal of Microbiological Methods. 2006; 66(1): 104-115. DOI: 10.16//j.mimet.2005.10.018
9. Bastianel C, Garnier-Semancik M, Renaudin J, Bové J-M, Eveillard S. Diversity of '*Candidatus* Liberibacter asiaticus', based on the *omp* gene sequence. Applied and Environmental Microbiology. 2005; 71(11): 6473-6478. DOI: 10.1128/AEM.71.11.6473-6478.2005
10. Teixeira DC, Eveillard S, Sirand-Pugnet P, Wulff A, Saillard C, Ayres AJ, et al. The *tuf*B-*sec*E-*nus*G-*rpl*KALJ-*rpo*B gene cluster of the liberibacters: sequence comparisons, phylogeny and speciation. International Journal of Systematic and Evolutionary Microbiology. 2008; 58: 1414-1421. DOI: 10.1099/ijs.0.65641-0
11. Teixeira DC, Danet J-L, Eveillard S, Martins Cintra de Jesús VJ, Yamamoto P, Lopes SA, *et al.* Citrus "huanglongbing" in Sao Paulo State, Brazil: PCR detection of the '*Candidatus* Liberibacter' species associated with the disease. Molecular and Cellular Probes. 2005; 19, 173-179. DOI: 10.1016/j.mcp.2004.11.002
12. Adkar-Purushothama CR, Quaglino F, Casati P, Ramanayaka JG, Bianco PA. Genetic diversity among '*Candidatus* Liberibacter asiaticus' isolated based on single nucleotide polymorphisms in 16S rRNA and ribosomal protein genes. Annals of Microbiology. 2002; 59(4): 681-688. DOI: 10.1007/BF03179208
13. Duan Y, Zhou L, Hall DG, Li W, Doddapaneni H, Lin H, *et al*. Complete genome sequence of citrus huanglongbing bacterium '*Candidatus* Liberibacter asiaticus' obtained through metagenomics. Molecular Plant Microbe Interactions. 2009; 22: 1011-1020. DOI: 10.1094/MPMI-22-8-1011
14. Lin H, Han CS, Liu B, Lou B, Bai X, Deng C, *et al*. Complete genome sequence of a Chinese strain of '*Candidatus* Liberibacter asiaticus'. Genome Announcements. 2013; 1: e00184-13. DOI: 10.1128/genomea.00184-13
15. Zheng Z, Sun X, Deng X, Chen J. Whole-genome sequence of '*Candidatus* Liberibacter asiaticus' from a "huanglongbing"-affected citrus tree in Central Florida. Genome Announcements. 2015; 3: e00169-15. DOI: 10.1128/genomea.00169-15
16. Chen J, Deng X, Sun X, Jones D, Irey M, Civerolo E. Guangdong and Florida populations of '*Candidatus* Liberibacter asiaticus' distinguished by a genomic locus with short tandem repeats. Phytopathology. 2010; 100: 567-572. DOI: 10.1094/PHYTO-100-6-0567
17. Katoh H, Subandiyah S, Tomimura K, Okuda M, Su HJ, Iwanami T. Differentiation of '*Candidatus* Liberibacter asiaticus' isolates by

variable-number tandem-repeat analysis. Applied and Environmental Microbiology. 2011; 77(5): 1910-1917. DOI: 10.1128/AEM.01571-10

18. Liu R, Zhang P, Pu X, Xing X, Chen J, Deng X. Analysis of a prophage gene frequency revealed population variation of '*Candidatus* Liberibacter asiaticus' from two citrus-growing provinces in China. Plant Disease. 2011; 95: 431-435. DOI: 10.1094/PDIS-04-10-0300
19. Wang X, Zhou C, Deng X, Su H, Chen J. Molecular characterization of a mosaic locus in the genome of '*Candidatus* Liberibacter asiaticus'. BMC Microbiology. 2012; 12: 18. DOI: 10/1186/1471-2180-12-18
20. Zheng Z, Bao M, Wu F, Van Horn C, Chen J, Deng X. A type 3 prophage of '*Candidatus* Liberibacter asiaticus' carrying a restriction-modification system. Phytopathology. 2018; 108: 454-461. DOI: 10.1094/PHYTO-08-17-0282-R
21. Wang X, Tan J, Bai Z, Su H, Deng X, Li Z, *et al.* Detection and characterization of Miniature Inverted-repeat Transposable Elements in '*Candidatus* Liberibacter asiaticus'. Journal of Bacteriology. 2013; 195(17): 3979-3986. DOI: 10.1128/jb.00413-13
22. Murray MG, Thompson WF. Rapid isolation of high molecular weight plant DNA. Nucleic Acids Research. 1980; 239: 487-491. DOI: 10.1093/nar/8.19.4321
23. Jagoueix S, Bové J-M, Garnier M. PCR detection of the two '*Candidatus* liberobacter' species associated with greening disease of citrus. Molecular and Cellular Probes. 1996; 10: 43-50. Disponible en: https://swfrec.ifas.ufl.edu/hlb/database/pdf/00000380.pdf
24. Zheng Z, Bao M, Wu F, Chen J, Deng X. Predominance of a single prophage carrying a CRISPR/cas system in '*Candidatus* Liberibacter asiaticus' strains in Southern China. PLoS ONE. 2016; 11(1): e0146422. DOI: 10.1371/journal.pone.0146422
25. Da Silva PA, Fassini CG, Sampaio LS, Dequigiovanni G, Zucchi MI, Wulff NA. Genetic diversity of '*Candidatus* Liberibacter asiaticus' revealed by short tandem repeats and prophage typing indicates population homogeneity in Brazil. Phytopathology. 2019; 109: 960-971. DOI: 10.1094/PHYTO-08-18-0295-R
26. Zheng Y, Huang H, Huang Z, Deng X, Zheng Z, Xu M. Prophage region and short tandem repeats of '*Candidatus* Liberibacter asiaticus' reveal significant population structure in China. Plant Pathology. 2021; 70(4): 959-969. DOI: 10.1111/ppa.13332

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