Identification of *Candidatus* Liberibacter asiaticus Associated with Huanglongbing Symptoms in the State of São Paulo, Brazil

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ABSTRACT. Plants showing Huanglongbing (HLB)-like symptoms were observed in the State of Sao Paulo, which is the largest citrus producing area in Brazil. The presence of "*Candidatus* Liberibacter" was verified in two of 10 leaf samples from symptomatic plants by PCR amplification with *Candidatus* Liberibacter specific primers OI1 and Oi2c. The sequences of both amplified products were identical and differed from the positive control sequence of "*Ca.* L. africanus" at 27 of 1,160 sites. BLAST searches yielded the highest scores with "*Ca.* L. asiaticus" sequences (GenBank accessions L22532, AY192576, AB008366 and AB038369, with identities ranging from 98.88 to 100%. Identities with other Liberibacter sequences varied from 94.71 to 97%. Analysis of 10 of 38 additional samples with HLB symptoms from different regions of São Paulo indicated the presence of *Ca.* L. asiaticus. These results confirmed the presence of the HLB agent in the State of São Paulo. This is a new disease for Brazil but the potential damage to citriculture in Brazil is enormous based on experience from other regions around the world. It remains unclear how and when the pathogen entered Brazil.

Huanglongbing (HLB), *ex* citrus greening, is one of the most serious diseases of citrus (2). It is caused by a phloem-limited, Gram-negative, non-culturable bacterium, designated "*Candidatus* Liberibacter", which is a member of the alpha-subdivision of the *Proteobacteria* (3). Genetic variants of *Candidatus* Liberibacter causing HLB have been identified (4). The African variant (designated "*Ca*. L. africanus") is heat-sensitive and vectored by *Trioza erytreae* (del Guercio) (Sternorrhyncha: Triozidae). The Asian variant, *Ca*. L. asiaticus, is heat-tolerant and vectored by *Diaphorina citri* Kuwayama (Sternorrhyncha: Psyllidae) (1). Occurrence of HLB has been restricted previously to some African and Asian countries, but in mid-2004, sweet orange plants in the State of São Paulo, Brazil, were found showing HLB-like symptoms of leaf mottling, chlorotic shoots and small, lopsided fruits.

To verify the presence of the HLB-associated bacterium by molecular analysis, petioles, leaf midribs and bark were collected from young branches of symptomatic plants. Tissue was ground in a mortar with liquid nitrogen and total DNA was extracted using the CTAB methodology of Murray and Thompson (7) with minor modifications. Polymerase chain reaction (PCR) amplification was carried out in 25 µl containing 2.5 µl of 10× buffer, 1.5 mmol $MgCl_2$, 2.5 mM of each dNTP, 12.5 ng each of primers OI1 and OI2c (5), 1 U of *Taq* DNA polymerase and varying volumes of total DNA extract. Amplification was performed in a PT 100 thermocycler (MJ Research, Waltham, MA, USA), with pre-denaturation at 94°C for 3 min, followed by 30 cycles at 94°C for 30 s, 60°C for 30 s and 72°C for 1 min. Amplification was concluded with a final cycle of denaturation at 94°C for 30 s, annealing at 60°C for 30 s, and extension at 72°C for 5 min. As a positive control for PCR we used DNA extracted from plants infected with *Ca.* L. africanus, kindly provided by Dr. Michael Luttig (*ARC LNR*—South Africa). Ten microliters of each PCR was loaded in a 1.2% agarose gel and DNA was visualized by staining with ethidium bromide.

Amplified DNA was purified using the MinElute Gel Extraction Kit (Qiagen, Inc., Valencia, CA, USA)

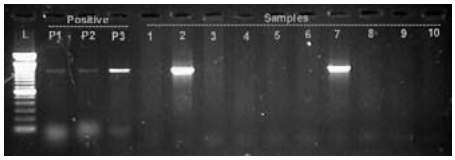

Fig. 1. Agarose gel electrophoresis of DNA amplified with primers Ol1 and Ol2c. Lane L indicates a 100 bp DNA size ladder. Lanes P1-P3 show DNA amplified from *Ca.* L. africanus positive controls. Lanes 1 to 10 show DNA amplified from samples 1-10.

and cloned into the pGEM-T vector (Promega Corporation, Madison, WI, USA) according to the manufacturer's instructions. *E. coli* competent cells were transformed by heat shock and plasmids were purified in 96-well plates using the boiling protocol (6). Sequencing was performed using the Big Dye terminator kit (Applied Biosystems, Foster City, CA, USA) according to the manufacturer's instructions. The sequences were obtained with an ABI 3730 DNA Analyzer (Applied Biosystems). Sequences were analyzed for *Xba*I endonuclease restriction sites using the MapDraw program in Lasergene99 (DNASTAR Inc., Madison, WI, USA). To confirm the presence of *Xba*I endonuclease sites, amplified DNA was digested with *Xba*I (Invitrogen Corp. Carlsbad, CA, USA).

A PCR product of approximately 1200 base-pairs (bp) was amplified from extracts of two of ten samples (Fig. 1). A fragment with the same electrophoresis mobility was obtained from the three positive controls (Fig. 1), corresponding to the product reported to have been amplified from *Ca.* L. asiaticus and *Ca.* L. africanus (5).

The sequences of fragments amplified from the positive samples #2 and #7 were submitted to Genbank and received the accession number AY919311. Nucleotide sequence similarity searches using the BLAST server (National Center for Biotechnology Information, U.S.

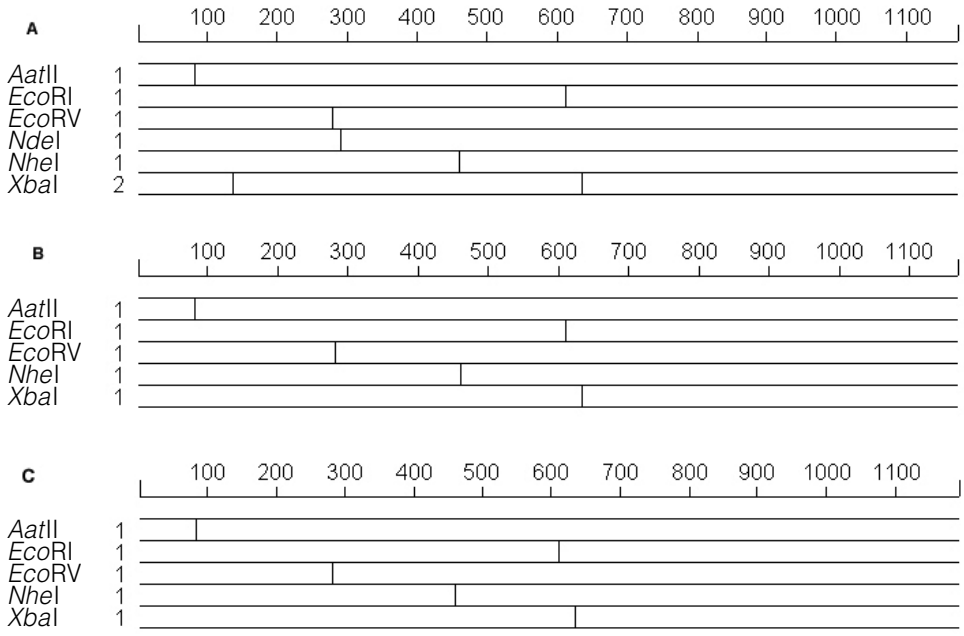

Fig. 2. Predicted maps of suspect HLB sequences digested with specific restriction endonucleases. A. Map for positive control, *Ca.* L. africanus. B. Restriction map for sample #2. C. Restriction map for sample #7. Column to the left of each map indicates specific restriction endonucleases tested.

National Library of Medicine, Bethesda, MD 20894) with sequence from samples #2 and #7 as query sequences showed high identity, 98.88 and 100% respectively, with Ca. L. asiaticus 16S ribosomal RNA gene sequences (Genbank accessions AB008366, AB038369, AY192576 and L22532). BLAST searches using the positive control sequence as the query sequence found high similarity with the partial sequence of the Ca. L. africanus 16S ribosomal RNA gene (Genbank accession L22533).

Differentiation of the two species can be done by *Xba*I digestion of amplified DNA (5). Digestion of the PCR product from *Ca.* L. africanus generates three fragments of 130, 506 and 520 bp, while that from *Ca.* L. asiaticus yields fragments of 520 and 640 bp. Computer analysis showed the presence of two *Xba*I restriction sites in the positive control sequence (Fig. 2A), as expected for *Ca.* L. africanus, and one *Xba*I restriction site for samples 2 and 7 (Fig. 2B, C). The predicted patterns (Fig. 2) were confirmed by *XbaI* digestion (Fig. 3) of control DNA and DNA from samples 2 and 7. Similar analyses of other samples with HLB-like symptoms from different regions of the State of São Paulo found *Ca*. L. asiaticus in 10 of 38 samples.

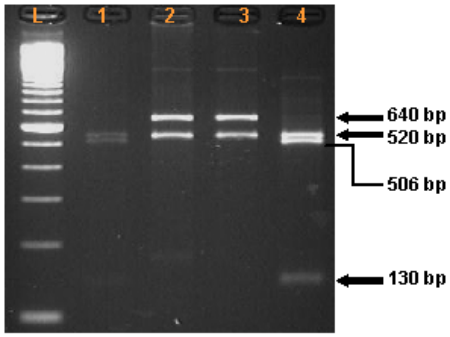

Fig. 3. Agarose gel electrophoresis of *Xba*1 digested DNA fragments amplified from *Ca.* L. africanus positive control (lanes 1 and 4) and HLB samples #2 (lane 2) and #7 (lane 3). Lane L 100 bp DNA size ladder.

This paper reports the first detection of a Liberibacter species in citrus in Brazil. Even though this pathogen appears to be a recent introduction, the potential damage it can cause to citriculture in Brazil is enormous as indicated by experience with HLB in other regions around the world. It remains unclear how and when the pathogen entered Brazil.

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