Detection of *Candidatus* Liberibacter asiaticus causing Citrus Huanglongbing disease

On-u-ma Ruangwong and Angsana Akarapisan*

Department of Plant Pathology, Faculty of Agriculture, Chiang Mai University, Chiang Mai 50200, Thailand

Ruangwong, O. and Akarapisan, A. (2006). Detection of *Candidatus* Liberibacter asiaticus causing Citrus Huanglongbing disease. Journal of Agricultural Technology 2(1): 111-120.

Detection of *Candidatus* Liberibacter spp. causing citrus Huanglongbing (HLB) disease was facilitated by taking samples from three different citrus orchards located in Chiang Mai, Chiang Rai, and Phrae provinces. A polymerase chain reaction (PCR) with specific primers OI1/OI2c and A2/J5 were used for detection and they produced specific band of 1160 bp and 703 bp, respectively. These were amplified from diseased leaves whereas no product from healthy citrus plants. To improve the method of detection for HLB disease, this study were a utilized five methods for pathogen DNA extraction from disease leaves and applied to a PCR; protocol three and four were most effective. When primers A2/J5 were used to identify the Liberibacter species, a 703 bp band was observed from all infected plants. The sequence of the bacterial ribosomal protein genes of samples from Chiang Mai (HLB-CM), Chiang Rai (HLB-CR), and Phrae (HLB-P) were analysed. From these results, it is apparent that the causal agent of citrus HLB disease, in these provinces, is *Candidatus* Liberibacter asiaticus.

Key words: *Candidatus* Liberibacter *asiaticus*, huanglongbing, greening, PCR, ribosomal protein gene

Introduction

Huanglongbing (HLB), commonly known as citrus greening is one of the most serious diseases that affects citrus fruit (Teixeira *et al*., 2004). HLB has destroyed an estimated 60 million trees in Africa and Asia (Timmer *et al*., 2003). It is caused by an uncultured phloem- limited bacterium that was first characterized in 1994 with the 16S rDNA sequence and shown to be a new genus in the α-Proteobacteria subdivision (Jagoueix *et al*., 1994). This bacterium was defined and named by Murray and Schleifer (1994) as '*Candidatus*' (a generic name given to all uncultured bacteria) 'Liberibacter'. However, two distinct species, '*Candidatus* Liberibacter asiaticus' and '*Candidatus* Liberibacter africanus' were described on the basis of gene

*Corresponding author: Angsana Akarapisan; e-mail: aangsana@chiangmai.ac.th

sequences from the rpl*KAJL*-rpo*BC* operon (*β*-operon) of Asia and Africa Liberibacters, respectively (Jagoueix *et al*., 1994). The detection of the pathogen is difficult because of its low concentration in its citrus hosts (McClean, 1970; Huang, 1979). The symptoms of HLB are like a zinc deficiency, often being confused with a nutritional problem (Timmer *et al*., 2003). This disease cannot easily be diagnosed by conventional procedures, such as electron microscopic examination of utra-thin sections, bioassay on indicator plants, or ELISA (Hung *et al*., 1999). A polymerase chain reaction (PCR) detection method, based on the amplification of 16S rDNA, was developed in 1996 (Jagoueix *et al*., 1996). A new PCR detection method based on the amplification of ribosomal protein genes, which allows for direct identification of both species by the size of the amplified DNA, was developed in 1999. This PCR method is as specific and at least as sensitive for the detection of both the two different Liberibacter species (Hocquellet *et al*., 1999).

In this study, the goal was to identify and confirm the pathogen causing citrus Huanglongbing disease in northern Thailand. In order to carry this out, it was necessary to develop a method for detection of the pathogen by using DNA extraction and PCR.

Materials and methods

Plant materials

Citrus cultivars Som Keowan and Chokun (Sainumphung) with characteristic symptom of HLB were used for this study. Leaf samples of Chokun were collected from citrus orchard in Ampher Wiangpapoa (Chiang Rai), Ampher Sansai, Ampher Maerim and Ampher Fang (Chiang Mai). Whilst Som Keowan were collected from Ampher Wangchin (Phare). Each leaf samples contained 20-40 leaves and were kept in plastic bags at -80°C before they were used for DNA extraction.

DNA extraction by CTAB method

DNA was isolated from 0.5 g of leaf midribs from infected or healthy plant by the CTAB (cetyltrimethylammonium bromide) method (Dellaporta *et al.,* 1983). PCR reaction was performed by using OI1/OI2c and A2/J5 primer to detect symptomatic leaves. The infected leaves were used for evaluation of DNA extraction.

Evaluation of DNA extraction

Protocol 1 (modified from Jagoueix *et al.*, 1996): Leaf midribs (0.5 g) were chopped to a fine mince and ground to a powder in 1.5 ml of 0.3M NaCl. This suspension was centrifuged for 15 min at 16,000 g. The pellets were resuspended in 25 µl of sterile water.

Protocol 2 (modified from Jagoueix *et al.* , 1996): Leaf midribs (0.5 g) were chopped to a fine mince and ground to a powder in 1.5 ml of TE buffer. The suspension was centrifuged for 15 minutes at 12,000 g. The supernatant was extracted with 0.5 ml of chloroform/isoamyl alcohol (24:1). The nucleic acids were precipitated by mixing equal volume of isopropanol followed by centrifugation at 14,000 g for 15 minutes. The pellets were washed with 70% ethanol, dried, and resuspended in 25 µl of sterile water.

Protocol 3 (modified from Hung *et al.*, 1999): Leaf midribs (0.5 g) were chopped into small pieces and ground in 4 ml of DNA extraction buffer. The suspension was incubated at 55°C for 1 hour and centrifuged at 4,000 g for 5 minutes. The supernatant was collected and added 0.125 volume of 5M NaCl and 10% CTAB in 0.7M NaCl. The mixture incubated at 65°C for 10 minutes and extracted by an additional two cycles of chloroform/isoamyl alcohol (24:1). The aqueous supernatant was collected and added 0.6 volume of isopropanol followed by centrifugation at 12,000 g for 10 minutes. The pellets were washed with 70% ethanol, dried, and resuspended in 25 µl of sterile water.

Protocol 4 (modified from Dellaporta *et al.*, 1983): Leaf midribs were cut into 0.5 g pieces and ground with 4 ml of cold grinding buffer. The extract was centrifuged at 10,000 g for 5 minutes at 4°C and collected supernatant. After centrifugation at 14,000 g for 25 minutes, the pellets were resuspended in 0.5 ml of CTAB buffer and incubated at 60°C for 30 minutes. The mixture was added 0.5 ml of chloroform/isoamyl alcohol (24:1) and centrifuged at 7,000 g for 5 minutes. The epiphase was collected and added an equal volume of isopropanol followed by centrifugation at 14,000 g for 15 minutes. The pellets were washed with 70% ethanol, dried, and resuspended in 25 µl of sterile water.

Protocol 5 (modified from Nakashima *et al.*, 1995): Leaf midribs were cut into 0.5 g pieces then 0.25 ml of CTAB solution was added, vortexed at 2 times/ 5 minutes and 0.25 ml of chloroform/isoamyl alcohol (24:1) added, followed by centrifugation at 15,000 g for 5 minutes. The supernatant was collected and precipitated by mixing 0.7 volume of isopropanol. After centrifugation at 15,000 g for 5 minutes, the pellets were dried and resuspended in 25 µl of sterile water.

Primers and PCR conditions

Specific primers, forward primer OI1 (5'-GCGCGTATGCAAGAGCGGCA-3') and reverse primer OI2c (5'-GCCTCGCGACTTCGCAACCCAT- 3'), were used for amplification of the 16S rDNA of the two *Candidatus* Liberibacter species. The PCR reaction was carried out in 25 µl of reaction mixture containing 1 µM of each primer, 0.2 mM of each four dNTPs, 1X PCR buffer, 2.5 mM $MgCl_2$, 0.5 units *Taq* DNA polymerase (Invitrogen) and 1 µl DNA template. The thermal cycle conditions were: one cycle at 94°C for 3 minutes; 35 cycles at 94°C for 60 seconds, 60°C for 30 seconds and 72°C for 90 seconds; followed by 72°C extension for 5 minutes. Reaction was carried out in a Programable Thermal Controller PTC-100^{TM} (MJ Research, USA).

To identify the two species, forward primer A2 (5'-TATAAAGGTTGACCTTTCGAGTTT-3') and reverse primer J5 (5'-ACAAAAGCAGAAATAGCA CGAACAA-3') defined from ribosomal protein gene of the rpl*KAJL*-rpo*BC* operon (*β*-operon) were used. The reaction mixture for PCR was performed using 25 µl of 1 µM of each primer, 0.2 mM of each four dNTPs, 1X PCR buffer, 2.5 mM $MgCl_2$, 0.5 units *Taq* DNA polymerase (Invitrogen) and 1 µl DNA template. Amplification was carried out in a Programable Thermal Controller PTC-100^{TM} (MJ Research, USA) with the following program: one cycle at 94°C for 3 minutes; 35 cycles at 92°C for 45 seconds, 62°C for 45 seconds and 72°C for 90 seconds; followed by 72°C extension for 5 minutes.

Analysis of PCR product

The PCR products were analyzed by gel electrophoresis using a 1% agarose in TE buffer (Tris base, boric acid and 0.5M EDTA [pH 8.0]) and stained with ethidium bromide. Gel was visualized and analyzed by the GEL documentation (SYNGENE; GENE Genious Bio Imaging System).

Sequencing

PCR products (HLB-CM from Chiang Mai, HLB-CR from Chiang Rai and HLB-P from Phare) from primers A2/J5 were sequenced according to the standard protocols for the ABI PRISM 3100 (version 3.7) automated DNA sequencer (Perkin Elmer) with ABI PRISM Ready Reaction Dye Termination Cycle Sequencing Kit. DNA sequences were compared to those in the NCBI

database using blastn. Multiple sequence alignments were performed with CLUSTAL W (1.82).

Results

PCR detection of Candidatus Liberibacter spp. in symptomatic leaves.

Primers OI1/OI2c were used for amplification of liberibacter 16S rDNA. An amplified product of the expected size (1160 bp) was observed from all symptomatic leaves. Fig. 1 shows that the amplified DNA on a 1% agarose gel, produced a band of 1160 bp from the positive control (lane 1) and DNA extracted from infected Chokun leaves (Sansai; Chiang Mai) (lane 2). No amplification was observed from healthy Chokun leaves (lane 3) or the negative control, water (lane 4). When primers A2/J5 were used, specific for the amplification of liberibacter ribosomal protein gene, band 703 bp was observed from all infected leaves. Fig. 2 shows that the amplified DNA on a 1% agarose gel, a band of 703 bp was obtained from positive control (lane 1) and infected Chokun leaves (Sansai; Chiang Mai) (lane 2). No amplification was obtained from healthy Chokun (lane 3) or the negative control, water (lane 4).

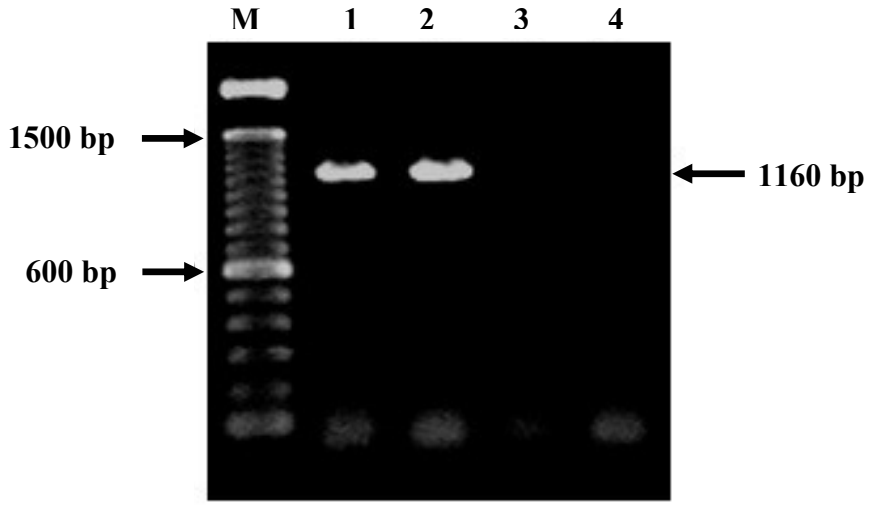

Fig. 1. Electrophoresis on 1% agarose gel of DNA amplified with primers OI1/OI2c from positive control (lane 1), DNA extracted from infected Chokun leaves (lane 2), healthy Chokun leaves (lane 3), and water (lane 4). M, 100 bp DNA ladder (Invitrogen).

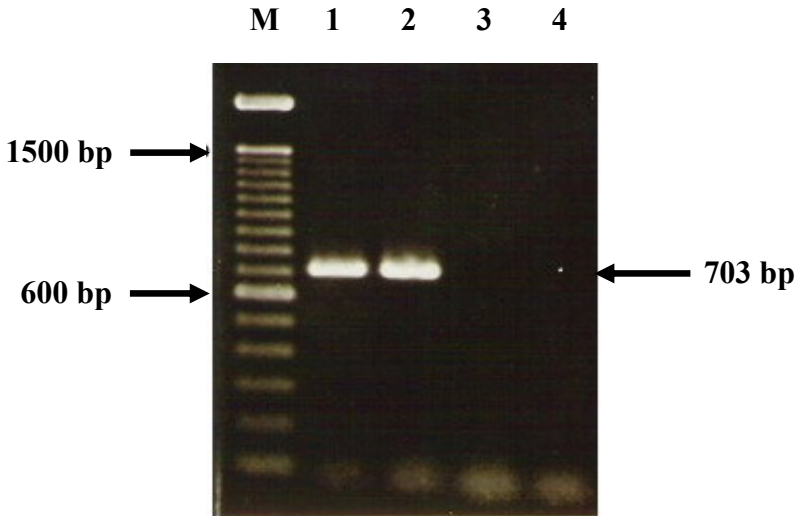

Fig. 2. Electrophoresis on 1% agarose gel of DNA amplified with primers A2/J5 from positive control (lane 1), DNA extracted from infected Chokun leaves (lane 2), healthy Chokun leaves (lane 3), and water (lane 4). M, 100 bp DNA ladder (Invitrogen).

Evaluation of DNA extraction

The quality of total DNA from each of the five protocols were displayed on a 1% agarose gel (Fig. 3).

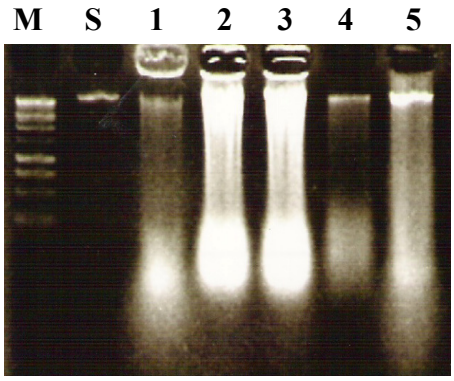

Fig. 3. Agarose gel electrophoresis of total DNA was extracted from infected citrus leaves with protocol 1, 2, 3, 4, 5 (lane 1, 2, 3, 4, 5). Lane M; 100 bp DNA marker (Invitrogen). Lane S; 100 ng of standard DNA

When PCR amplification with specific primers OI1/OI2c, no amplified DNA could be observed on the gel from isolated DNA with protocol 1 and 2 (Fig. 4; lane 1 and 2). Amplification of a 1160 bp also occurred when DNA was extracted with protocol 3, 4 and 5 (Fig. 4; lane 3, 4 and 5) but a weak band was observed from protocol 5. Similarly, no DNA band was obtained from protocol 1 and 2 (Fig. 5; lane 1 and 2) with primers A2/J5. A band of 703 bp was obtained from protocol 3, 4 and 5 (Fig. 5; lane 3, 4 and 5). Comparison of a time for DNA extraction; protocol 1, 2, 3, 4 and 5 can be completed within 30 min, 2 h, 3 h and 30 min, respectively.

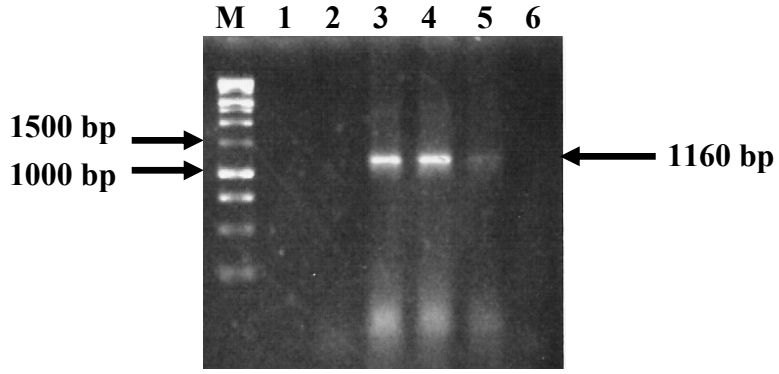

Fig. 4. Electrophoresis on 1% agarose gel of DNA amplified with primers OI1/OI2c from DNA extracted from infected Chokun leaves following protocol 1, 2, 3, 4, 5 (lane 1, 2, 3, 4, 5), and water (lane 6) M, 100 bp DNA marker (Invitrogen).

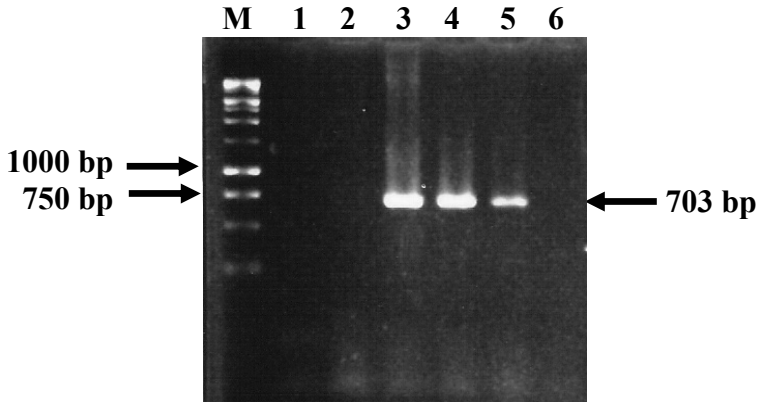

Fig. 5. Electrophoresis on 1% agarose gel of DNA amplified with primers A2/J5 from DNA extracted from infected Chokun leaves following protocol 1, 2, 3, 4, 5 (lane 1, 2, 3, 4, 5), and water (lane 6) M, 100 bp DNA marker (Invitrogen).

Partial sequence of the ribosomal protein gene of the Candidatus Liberibacter spp. with primers A2/J5

The three partial sequences, HLB-CM, HLB-CR and HLB-P, of the ribosomal protein gene obtained after amplification with primers A2/J5 were similar. The results showed that each sequence consisted of 608 bp and encode 164 amino acid (Fig. 6). When these sequences were compared with the known Liberibacter species in GenBank, sequences homology were higher with *Candidatus* Liberibacter asiaticus (100%) (GenBank accession numbers AY342001, AY266352 and M94319) than with *Candidatus* Liberibacter africanus (75%) or *Candidatus* Liberibacter asiaticus subsp. capensis (70%).

```
  1   ctcttgtttagttgtttttgtgtggattccttttcgctatcg
      -  -  -  -  -  -  -  -  -  -  -  -  -  -  -
 45   gatcgcttctttttgtaagggatgcgttaggatttttgttcttc
      -  -  -  -  -  -  -  -  -  -  -  -  -  -  -
 90   ttcgaaatcaagatatgaaaatattttcttggtatagatatagga
      -  -  -  -  -  -  -  -  -  -  -  -  -  -  -
135   aaaggaatgggtatatttgtcatctggagatgaaagttgaataga
      -  -  -  -  -  -  -  -  -  -  -  -  -  M  N  R
180   caaggaaagagcgtagaaatttctgaattaagtaagatttttct
      Q  G  K  S  V  E  I  S  E  L  S  K  I  F  S
225   tcttctggatcaattgttgttgcacattataagggaattagtgtt
      S  S  G  S  I  V  V  A  H  Y  K  G  I  S  V
270   gcgcaaattaaagatcttcggaaaaagatgcgggaagctggtgga
      A  Q  I  K  D  L  R  K  K  M  R  E  A  G  G
315   ggtgtaaaagttgccaaaaatcgtctcgtcaagattgctatccgt
       G  V  K  V  A  K  N  R  L  V  K  I  A  I  R
360   gatactagtattagaggaatatctgatctttcgtgggcagtctc
       D  T  S  I  R  G  I  S  D  L  F  V  G  Q  S
405   taattgtctattcggatagtcctgttattgctccttaaaatttcg
      L  I  V  Y  S  D  S  P  V  I  A  P  K  I  S
450   gttagcttttcaaatgacaataatgaatttagagttcttggtggg
      V  S  F  S  N  D  N  N  E  F  R  V     L  G  G
495   gttgtagagaagggcgtccttaatcaagattctatcaagcaaatt
      V  V  E  K  G  V  L  N  Q  D  S  I  K  Q  I
540   gcttctttacccgatcttgagggtattcgagctggtatcataagt
      A  S  L  P  D  L  E  G  I  R  A  G  I  I  S
585   gctatccaatctaatgcaactaga
      A  I  Q  S  N  A  T  R
```

Fig. 6. Partial nucleotide sequence (normal letter) of the ribosomal protein gene (*rpl*J) and encode amino acid sequence (bold letter) from HLB-CM, HLB-CR and HLB-P.

Discussion

The five protocols of DNA extraction were modified and applied to PCR with specific primer OI1/OI2c and A2/J5 for the detection of *Candidatus* Liberibacter spp. Protocol three modified from Hung *et al.* (1999) and protocol four modified from Dellaporta *et al.* (1983) were most effective. When amplification was performed on these DNA extractions, a band of 1160 bp and 703 bp were observed from primer OI1/OI2c and A2/J5, respectively. However, the most rapid and least expensive protocol for DNA extraction was protocol three. Protocol one and two were not effective because they could not produce the specific bands. Protocol five could produced specific fragments but they were weak and poorly band for primers OI1/OI2c and A2/J5, because it was not enough liberibacter DNA extracted for detection. The size of the amplified DNA of primers A2/J5 is smaller than OI1/OI2c, thus the target degradation is less critical (Hocquellet *et al.*, 1999). Therefore, the sensitivity of primers A2/J5 was the same or slightly better compared with primers OI1/OI2c.

In order to distinguish between *Candidatus* Liberibacter asiaticus and *Candidatus* Liberibacter africanus, primer A2/J5 was used. The amplification of the ribosomal protein genes allows for a direct identification of the two species by comparing the size of the DNA band. This primers allows for a specific amplification of 703 bp from *Candidatus* Liberibacter asiaticus whereas the *Candidatus* Liberibacter africanus produces a band of 669 bp (Hocquellet *et al*., 1999). When these primers were used to identify the liberibacter on the samples from HLB-CM (Chiang Mai), HLB-CR (Chiang Rai) and HLB-P (Phrae), a band of 703 bp was obtained from all infected plants. No amplification was observed from water or healthy citrus plants. The band 703 bp was sequenced and compared with data from GenBank. The results indicated that the sequence was closely related to the record of *Candidatus* Liberibacter asiaticus, since they were a part of the ribosomal protein gene (*rplJ*). It can be concluded that the causal agent of citrus Huanglongbing (greening) disease in Chiang Mai, Chiang Rai and Phrae provinces is *Candidatus* Liberibacter asiaticus.

The detection of HLB disease in young citrus plants is important to prevent a wide- spread outbreak of this disease. The sensitivity PCR assay is an excellent method for detecting fastidious bacteria in its host (Hung et al., 1999). Thus, it is recommended that DNA extraction from protocol three and PCR with specific primers A2/J5 be utilized for the detection of HLB disease and the identification of the two Liberibacter species.

Acknowledgements

This research was supported by Subproject: Graduate study and research in agricultural biotechnology, Faculty of Agriculture, Chiang Mai University.

References

Dellaporta, S.L., Wood, J. and Hicks, J.B. (1983). A Plant DNA minipreparation : version II. Plant Molecular Reporter 4: 19-20.

Hocquellet, A., Toorawa, P., Bové, J.M. and Garniner, M. (1999). Detection and identification of the two *Candidatus* Liberobacter species associated citrus huanglongbing by PCR amplification of ribosomal protein genes of the operon. Molecular and Cellular Probes 13: 373-379.

Huang, CH. (1979). Distribution of likubin pathogen in likubin affected citrus plant. Journal of Agricultural Research of China 28: 29-33.

Hung, T.H., Wu, M.L. and Hong-J, S. (1999). Development of a rapid method for the diagnosis of Citrus Greening Disease using the Polymerase Chain Reaction. Journal Phytopathology 147: 599-604.

Jagoueix, S., Bové, J.M. and Garnier, M. (1994). The phloem-limited bacterium of Greening is a member of the alpha subdivision of the proteobacteria. *International* Journal of Systemic Bacteriology 44: 379-386.

Jagoueix, S., Bové, J.M. and Garnier, M. (1996). PCR detection of the two 'Candidatus' liberobacter species associated with greening disease of citrus. Molecular and Cellular Probes 10: 43-50.

McClean, A.P.D. (1970). Greening disease of sweet orange: its transmission in propagative parts and distribution in partially disease trees. Phytophylactica 2: 263-268.

Murray, R.G.E. and Schleifer, K.H. (1994). Taxonomic notes: a proposal for recording the properties of putative taxa of procaryotes. International Journal of Systematic Bacteriology 44: 174-176.

Nakashima, K., Promintara, M., Ohtsu, Y. and Kano, T. (1995). Improve detection of 16S rDNA of the Thai Isolate of GO. In: *Development of Methods for the Diagnosis and Control of Citrus Greening Disease in Thailand*. Under the cooperation research program between Thailand and Japan: 33-46.

Teixeira, D.D.C., Danetb, J.L., Eveillardb, S., Martinsa, E.C., Juniora, W.C.J., Yamamotoa, P.T., Lopesa, S.A., Bassanezia, R.B., Ayresa, A.J., Saillardb, C. and Bove, J.M. (2004). Citrus huanglongbing in Sao Paulo State, Brazil: PCR detection of the "Candidatus" Liberibacter species associated with the disease. Molecular and Cellular Probes 19: 173–179.

Timmer, L.W., Garnsey, S.M. and Broadbent, P. (2003). Diseases of *Citrus*. In: *Disease of Tropical Fruit Crops* (ed. R.C. Ploetz). APS Press, USA: 163-195.

(Received 4 January 2006; accepted 14 May 2006)