INFECTION OF WAMPEE AND LEMON BY THE CITRUS HUANGLONGBING PATHOGEN (*CANDIDATUS* LIBERIBACTER ASIATICUS) IN CHINA

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SUMMARY

Single-step and nested polymerase chain reactions (PCR) were used to determine the presence of *Candidatus* Liberibacter asiaticus, the phloem-limited bacterial pathogen of Huanglongbing (HLB), in leaves of wampee [*Clausena lansium* (Lour.) Skeels], lemon [*Citrus limon* (L.) Burm.] and several other citrus species. Specific PCR products were obtained when single-step PCR and nested PCR were used to analyze wampee and lemon samples with or without visible HLB symptoms. Nested-PCR was found to be more accurate than single-step PCR with a sensitivity of about 10^4 times higher. The amount of bacterial DNA was positively correlated with HLB symptoms in the leaves. Amplicons from single-step PCR and those from nested PCR showed 100% and 99% identity, respectively, with sequences of the *rpl*KAJL-*rpo*BC gene cluster of *Ca.* Liberibacter asiaticus. This pathogen is therefore able to infect wampee and lemon, in the leaves of which it can be readily detected by PCR. This is the first report of infections by *Ca*. L. asiaticus in wampee.

Key words: Huanglongbing, greening, *Candidatus* Liberibacter asiaticus, citrus, lemon, wampee, diagnosis, single-step PCR, nested PCR.

INTRODUCTION

Huanglongbing (HLB), previously known as 'greening', is one of the most severe diseases of citrus, including orange, mandarin, grapefruit, lemon and lime in Asia, south-east Asia, south and east Africa and the Arabian Peninsula (Da Graça, 1991; Garnier and Bové, 1993, 1996). HLB is caused by a phloem-limited, non cultured, gram-negative bacterium (Garnier *et al.*, 1984), which, based on the sequence of its 16S rDNA (Jagoueix *et al.*, 1994), the 16S/23S ribosomal intergenic region (Jagoueix *et al.*, 1997) and the *rpl*KAJL-*rpo*BC gene cluster (®A-operon) (Villechanoux *et al.*, 1993; Planet *et al.*, 1995), proved to represent a new genus, the genus Liberibacter, in the a-subdivision of the *Proteobacteria*, and was assigned a *Candidatus* status as *Candidatus* Liberibacter, according to the rules established for non cultured microorganisms (Murray and Schleifer, 1994).

Greening organisms present in Asia and Africa were shown to be different species that were named *Candidatus* Liberobacter asiaticum and *Candidatus* Liberobacter africanum, respectively (Jagoueix *et al.*, 1994, 1997), later changed to *Candidatus* Liberibacter asiaticus and *Candidatus* Liberibacter africanus (Garnier *et al*., 2000). The HLB pathogen occurring in China is classified as *Candidatus* Liberibacter asiaticus, and is distributed in 11 provinces including Guang Dong, Guang Xi, Zhe Jiang, Fu Jian, Tai Wan, and Hai Nan (Ding *et al.*, 2004). To give an idea of the severity and economical importance of HLB in the groves of the Yang-Cun Oversea Citrus Research Institute in Guang Dong province, 378,000 infected trees were uprooted between 1993 and 1995 (Zhuang, 2003). To minimize unexpected losses to the developing Chinese citrus industry, investigations on the distribution of HLB using sensitive and reliable detection methods are therefore of utmost importance.

Two members of the family *Rutaceae*, wampee [*Clausena lansium* (Lour.) Skeels] and lemon [*Citrus limon* (L.) Burm.] are two popular species grown in tropical and subtropical China.

Their cultivation is expanding (Weng, 1997; Li, 2004) and their commercial value is much higher than that of other rutaceous species, wampee in particular (Weng, 1997). To the best of our knowledge there is no previous report of HLB infections in wampee, but lemon was shown to be infected by *Ca.* L. asiaticus in Nepal using DNA hybridization (Regmi *et al*., 1996), in Indonesia, Cambodia, Laos and Myanmar using PCR (Bové *et al*., 2000, Garnier and Bové, 2000), and by *Ca.* L. africanus in South Africa using PCR (Garnier *et al*., 2000). In the course of recent surveys symptoms resembling those induced by HLB were observed in Guang Dong on trees of wampee and lemon, i.e. small-sized

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Table 1. Citrus accessions from which samples were collected for PCR testing. All samples gave a positive reaction in single-step PCR, except for those marked by an asterisk, which were positive in nested PCR.

Accessions naturally infected by HLB (positive controls)	No. of samples from trees with yellowing	No. of samples from trees with blotchy mottling	No. of samples from trees with zinc deficiency-like symptoms	No. of samples with no obvious symptoms	Totals
Ponkan 1[a]	15	10	12	-	37
Ponkan 2[b]	18	18	15	-	51
ZaGan	13	9	6	-	28
Hongjiang sweet orange	21	17	11	-	49
Anliu sweet orange	20	5	4	-	29
Luo Gang sweet orange	28	22	5	-	55
Periwinkle	16	0	0	-	16
Accessions checked for the presence of the HLB agent					
Wampee	2	0	0	17*	19
Rough lemon	1	3	0	12*	16
Eureka lemon	2	1	0	15*	18

[a] From Yang Cun Oversea Citrus Research Institute
[b] From the Agrotechnical Station of Luo Gang, Guangzhou
- No samples collected.

leaves with vein yellowing and blotchy mottling of the blades, but no zinc deficiency–like symptoms, which are sometimes associated with the disease (Zhao, 1981; Da Graca, 1991). The possibility that these symptoms could be elicited by the same agent causing HLB in other citrus species, suggested carrying out specific investigations to ascertain their nature.

MATERIALS AND METHODS

Plant material. Plant materials used in these studies (Fig. 1 and Table 1) were symptomatic leaves of wampee (*Cl. lansium*), rough lemon (*Citrus jambhiri* Lush), and Eureka lemon (*C. limon*) from the Institute of Fruit Tree Research, Guang Dong Academy of Agricultural Science. HLB positive controls were naturally infected Ponkan (*Citrus reticulata* Blanco), Anliu sweet orange (*Citrus sinensis* Osb.), Hongjiang sweet orange (*C. sinensis* x *C. reticulata* hybrid), Tsunokaori, experimentally infected periwinkle (*Catharanthus roseus*) obtained from Yang Cun Oversea Citrus Research Institute, Luo Gang sweet orange and Ponkan from the Agrotechnical Station of Luo Gang, Guangzhou. Negative controls were leaves of healthy trees of the same species and varieties as above that were grown in an insect-proof greenhouse.

Genomic DNA isolation. Total genomic DNA was isolated with a previously described procedure (Yi *et al.*, 1999; Ding *et al.*, 2004) with slight modifications. Approximately, 0.3 g of chopped midribs were frozen in liquid nitrogen, and then quickly ground to a fine powder with mortar and pestle. The powder was collected into a 1.5 ml Eppendorf tube with 1 ml of preheated (65°C) CTAB (Cetyltrimethyl Ammonium Bromide) extraction buffer (100 mM Tris-HCl, 1.4 M NaCl, 20 mM EDTA, 2% CTAB, 1% PVP, 0.2% β-mercaptoethanol). Samples were incubated at 65°C for 1 h, mixed with an equal volume of 24:1 chloroform-isoamyl alcohol mixture, and centrifuged at 12,000 rpm for 10 min. The upper phase was recovered, mixed with an equal volume of isopropanol and placed at -20°C. Precipitated DNA was sequentially washed with 75% and 95% ethanol, air dried, and resuspended in 100 µl of double distilled water.

The extracted genomic DNA was used as template for single-step PCR and nested-PCR reactions using *Ca.* L. asiaticus-specific PCR primers (Kong *et al.*, 2000; Li and Ke, 2002) for amplification of *rpl*KAJL-*rpo*BC gene cluster (b-operon) sequences (Villechanoux *et al.*, 1993).

PCR assays. Single-step and nested PCR reactions were done in a 25 µl volume with reaction mixtures containing 2.5 µl of 10x PCR buffer, 0.1 mM of each dNTP, 0.4 mM of each primer, one unit of Taq DNA Polymerase (TaKaRa, Dalian, China), and corresponding templates.

Templates were 10 ng of genomic DNA for single-step PCR and 1 µl of the first PCR product for nested-PCR. The primer set used for single-step PCR was F1 (forward) and R1 (reverse) (Table 2). The second primer set F2 (forward) and R2 (reverse) together with F1 (forward) and R1 (reverse) were used for nested PCR (Table 2).

Table 2. Primer sequences used in this work, designed on GenBank sequence M94319.

Single-step PCR primers/Nested-PCR outer primers	F1: 5'-TGAATTCTTCGAGGTTGGTGAGC-3' R1: 5'-AGAATTCGACTTAATCCCCACCT-3'	Homologous with nt 39-61 Complementary to nt 573-551
Nested-PCR inner primers	F2: 5'-GCGTTCATGTAGAAGTTGTG-3' R2: 5'-CCTACAGGTGGCTGACTCAT-3'	Homologous with nt 134-153 Complementary with nt 533-514

The cycling parameters of single-step PCR were: 95°C for 3 min, followed by 35 cycles consisting of denaturation at 94°C for 30 s, annealing at 53°C for 30 s and extension at 72°C for 1 min, and final extension at 72°C for 10 min. Similar conditions were used for nested PCR except for the annealing temperature was 55°C. Both PCR types were carried out in a 60-well block PCR Thermal Cycler (Model PTC-100, MJ Research, USA).

The amplified PCR products were separated by electrophoresis on 1.5% agarose gels (1x TBE buffer) with ethidium bromide staining (0.5 µg ml^{-1}). The gel was visualized under UV light, and the size of PCR products was determined with a molecular marker (100 bp DNA Ladder Marker, Dingguo, China).

DNA cloning and sequencing. The expected PCR products were recovered and purified using PCR Fragment Recovery Kit (TaKaRa, Dalian, China), inserted into pGEM-T vector, which was transformed into competent *E. coli* JM109 cells. Bacterial colonies were selected on LB plates with Amp/IPTG/X-gal. Positive plasmid recombination was checked by PCR, and the sequence was determined by a commercial sequencing service (Shanghai Sangon Biological Engineering & Technology and Service Co. Ltd, Shanghai, China).

RESULTS

Single-step PCR. An amplicon of 535 bp in size was obtained with the *Ca.* L. asiaticus-specific primer pair F1-R1 (Fig. 2) from symptomatic leaves of all HLB-diseased citrus accessions, including Ponkan and Hongjiang sweet orange from Yang Cun (lane 2 and 4), Anliu sweet orange (lane 5), ZaGan (lane 6), infected Periwinkle (lane 7), sweet orange from Luo Gang (lane 8), Ponkan from Luo Gang (lane 9), Rough lemon (lane 11), Eureka lemon (lane 12), Wampee (lane 13). By contrast, there was no amplification from leaf extracts of healthy Ponkan (lane 10), healthy Eureka lemon (lane 14), and water control (lane 3).

Since a faint band was observed in gels of some samples from symptomless leaves of wampee, rough lemon and Eureka lemon, PCR assays were repeated at least three times with these samples obtaining consistent positive results. To establish whether these symptomless leaves were actually infected, a more sensitive assay as that afforded by nested-PCR was tried.

Nested PCR. The use in nested PCR assays of primer pair F2 and R2 (Table 2) together with primer pair F1 and R1, designed on the published sequence of *Ca.* L. asiaticus (Villechanoux *et al*., 1993) allowed the clear-cut amplification of a 400 bp product from symptomless leaves of wampee, eureka lemon, and rough lemon (Fig. 3, lanes 4, 5 and 6) as well as from symptomatic leaves of infected Ponkan from Yang Cun, Eureka lemon, and wampee (Fig. 3, lane 2, 8, and 9). No amplicons were obtained from the water control and from leaves of healthy wampee (Fig. 3, lanes 3 and 7). Thus, the HLB agent seems to be detectable also in symptomless leaves, if a nested-PCR approach is followed.

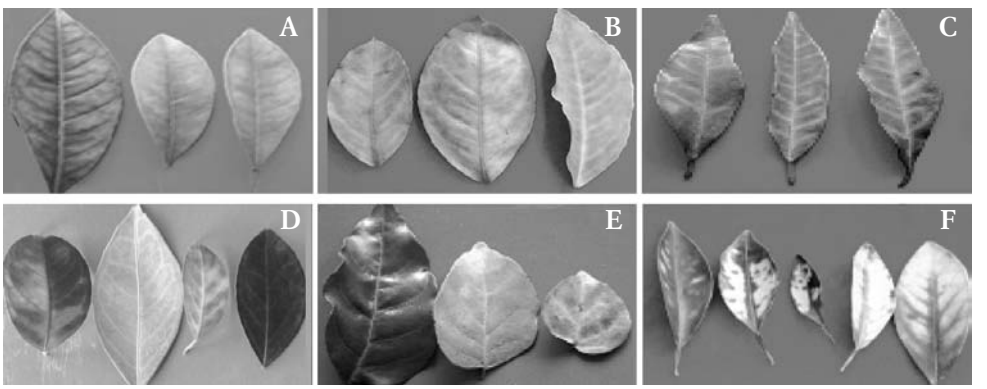

Fig. 1. Yellowing and blotchy mottling induced by HLB in the leaves of different citrus species. (**A**) Rough lemon (healthy leaf on the left). (**B**) Eureka lemon. (**C**) Tsunokaori. (**D**) Sweet orange (healthy leaf on the extreme right). (**E**) Wampee (healthy leaf on the left). (**F**) Ponkan.

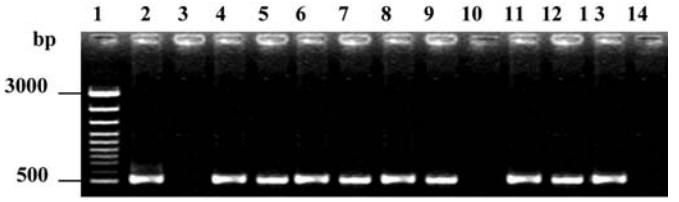

Fig. 2. Results of single-step PCR using primers pair F1/R1 (see Table 2) that amplifies a 535 bp DNA fragment. 100 bp DNA Ladder IV (lane 1); water control (lane 3); positive controls in lanes 2 (Ponkan), 4 (Hongjiang sweet orange), 5 (An Liu sweet orange), 6 (Tsunokaori), 7 (Periwinkle), 8 (sweet orange from Luo Gang), 9 (Ponkan from Luo Gang); healthy Ponkan control (lane 10); symptomatic rough lemon (lane 11), Eureka lemon (lane 12), wampee (lane 13); symptomless wampee in lane 14.

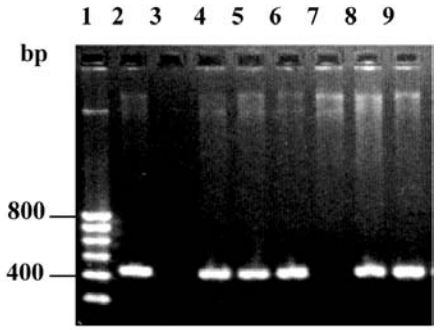

Fig. 3. Results of nested PCR using primers pair F1/R1 and F2/R2 (see Table 2) that amplify a 400 bp DNA fragment. 100 bp DNA Ladder II (lane 1); water control (lane 3); symptomatic Ponkan (lane 2); infected but symptomless wampee (lane 4), Eureka lemon (lane 5), rough lemon (lane 6); healthy wampee (lane 7); symptomatic Eureka lemon (lane 8); symptomatic wampee (lane 9).

Comparative sensitivity of single-step versus nested PCR. To comparatively assess the sensitivity of single-step and nested-PCR, a serial assay was carried out. Whereas the detection limit of genomic DNA of single-step PCR was about 1 to 10 pg in our assay system, nested-PCR detected up to 1 fg DNA (Fig. 4), thus proving to be at least 10^4 times more sensitive than conventional PCR. It ensued that samples which were negative or weakly positive in single-step PCR were clearly positive in nested-PCR. In fact, the totality of 53 leaf samples of wampee (19), Eureka lemon (18), and rough lemon (16) were found positive by nested-PCR, whereas only 13% of them reacted in single-step PCR. This high sensitivity may not be the consequence of false positives because no amplification was ever obtained from negative controls.

DNA cloning and sequencing. The 535 bp and the 400 bp products amplified by single-step and nested PCR, respectively, were successfully cloned and sequenced. When these sequences were analyzed using the BLAST program at the National Center for Biotechnology Information (http://www.ncbi.nlm.nih.gov) they were found to share 100% and 99% nucleotide identity with comparable DNA fragments from the *rpl*KAJL-*rpo*BC gene cluster (β-operon) of *Ca* L. asiaticus (GenBank accession number M94319 from Villechanoux *et al*., 1993). This was taken as evidence that the HLB agent was indeed present in wampee and lemon leaves with vein yellowing and blotchy-mottling and in symptomless samples.

DISCUSSION

Wampee and lemon are becoming increasingly popu-

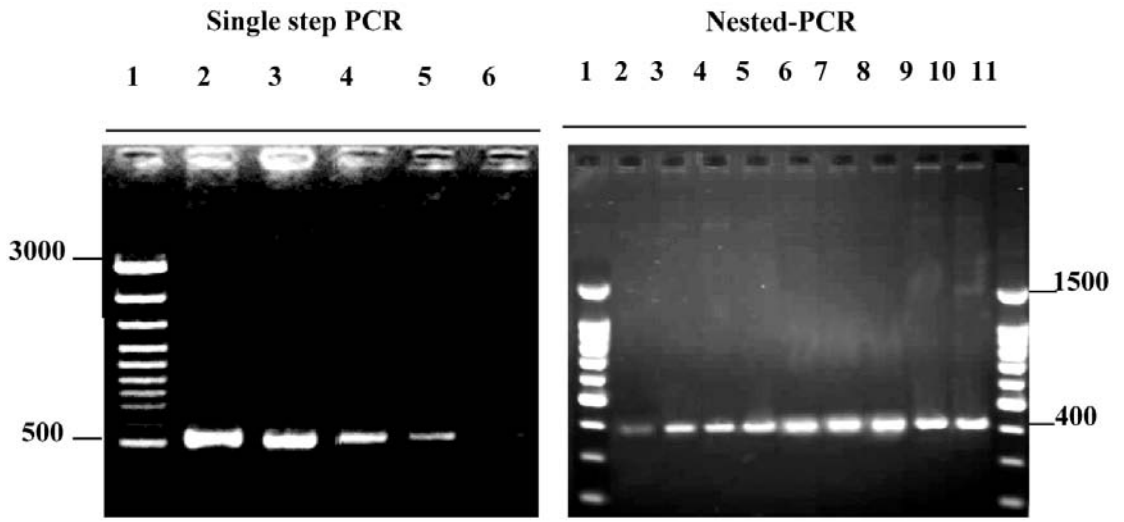

Fig. 4. Detection threshold of *Ca.* Liberibacter asiaticus genomic DNA by single-step and nested PCR. 100 bp DNA Ladder IV in left panel lane 1 and 100 bp DNA Ladder II in lane 1 and lane 11 of the right panel. Left panel shows detection by single-step PCR: 10 ng DNA (lane 2), 1 ng (lane 3), 100 pg (lane 4), 10 pg (lane 5), 1 pg (lane 6). Right panel shows detection by nested PCR: 0.1 fg DNA (lane 2), 1 fg (lane 3), 10 fg (lane 4), 100 fg (lane 5), 1 pg (lane 6), 10 pg (lane 7), 100 pg (lane 8), 1 ng (lane 9), 10 ng (lane 10).

lar in southern China, where their cultivation is rapidly expanding because of the higher commercial value of their crop as compared with that of other citrus species. Wampee is not known to be host of HLB, one of the destructive diseases of citrus, induced by the phloem-limited bacterium *Ca.* L. asiaticus and infection of lemon by the same agent has not been reported from China. For this reason, the presence in Guang Dong of wampee, as well as lemon trees showing symptoms strongly recalling those of HLB caught our attention and prompted us to carry out investigations for identifying the possible agent of the disease.

PCR is regarded as one of the most sensitive and accurate methods for detection of various kinds of plant pathogens. In this study, we used two *Ca.* L. asiaticus-specific PCR primer pairs for single-step PCR and nested-PCR, and successfully detected the HLB pathogen in symptomatic wampee, and citrus cultivars and hybrids including lemon, Ponkan, ZaGan and Hongjiang sweet orange. These results were consistent with those of previous studies (Kong *et al.*, 2000; Li and Ke, 2002).

The detection threshold of single-step PCR from symptomatic leaves was 10 pg of bacterial DNA, which shows the considerable potential of this type of assay as a reliable procedure for identifying the HLB agent in infected symptomatic plant tissues. However, as reported also by Li (2002) nested-PCR had a higher sensitivity and made it possible the successful detection of the pathogen in leaves without visible symptom. Using this type of assay we detected bacterial DNA in the leaves of all trees, regardless of whether or not they showed symptoms. The lowest DNA quantity that could be detected with our protocol was about 1 fg, which is in agreement with other studies reporting detection of less than 25 pg or 1 fg of pathogen DNA in soil or plant materials (Tsushima *et al.*, 1995; Bonants *et al.*, 1997). These results indicate that the nested PCR approach enables specific detection of very low levels of target bacterial DNA and can therefore be used as an early warning detection tool for identifying the HLB agent in citrus plants that do not show yet obvious symptoms. The experimental evidence obtained with the present study represents the first world report of *Ca.* L. asiaticus in wampee and its first record in lemon in China.

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REFERENCES

Bonants P., Hagenaar-de Weerdt M., van Gent-Pelzer M., Lacourt I., Cooke D., Duncan J., 1997. Detection and identification of *Phytophthora fragariae* Hickman by the polymerase chain reaction. *European Journal of Plant Pathology* **103**: 345-355.

Bové J.M., Erti Dwiastuti M., Triviratno A., Supriyanto A., Nasli E., Becu P., Garnier M., 2000. Incidence of Huanglongbing and citrus rehabilitation in North Bali, Indonesia. In: da Graça J.V., Lee R.F., Yokomi R.K. (eds.). *Proceeding of the 14th Conference of the International Organization of Citrus Virologists*, *Riverside* 2000, 200-206.

Da Graca J.V., 1991. Citrus greening disease. *Annual Review of Phytopathology* **29**: 109-136.

Ding F., Yi G.J., Wang G.P., 2004. Research on the PCR and Nested-PCR detection of Citrus Huanglongbing pathogen. *Acta Horticulturae Sinica* **31**(6): 803-806.

Ding F., Yi GJ., Wang GP., 2003. Advances in Research on Citrus Huanglongbing, *Guangdong yuanyi* **4**: 60-62.

Garnier M., Bovè JM., 1993. Citrus greening disease and the greening bacterium. In: Moreno P., da Graça J.V., Timmer L.W. (eds.). In: *Proceedings of the 12th Conference of the International Organization of Citrus Virologists, Riverside* 1993, 212-219.

Garnier M., Bové J.M., 1996. Distribution of the Huanglongbing (greening) Liberobacter species in fifteen African and Asian countries. In: da Graça J.V., Moreno P., Yokomi R.K. (eds.). *Proceedings of the 13th Conference of the International Organization of Citrus Virologists Riverside* 1996, 388-391.

Garnier M., Bové J.M., 2000. Huanglongbing in Cambodia, Laos and Myanmar. In: J.V. da Graça, R.F. Lee, R.K. Yokomi (eds.). *Proceedings of the 14th Conference of the International Organization of Citrus Virologists, Riverside* 2000, 378-380.

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, *Calodendron capense*, in the Western Cape province of South Africa, Proposal of "*Candidatus* Liberibacter africanus subsp. Capensis". *International Journal of Systematic and Evolutionary Microbiology* **50**: 2119-2125.

Garnier M., Danel N., Bovè JM., 1984. Aetiology of citrus greening disease. *Annals of Microbiology* **135A**: 169-179.

Jagoueix S., Bovè J.M., Garnier M., 1994. The phloem-limited bacterium of greening disease is a member of the α-subdivision of the *Proteobacteria*. *International Journal of systematic Bacteriology* **44**: 379-386.

Jagoueix S., Bovè J.M., Garnier M., 1997. Comparison of the 16S/23S ribosomal intergenic region of '*Candidatus liberobacter asiaticum*' and '*Candidatus liberobacter africanum*', the two species associated with citrus Huanglongbing (greening) disease. *International Journal of Systematic Bacteriology* **47**: 224-227.

Kong W.W., Deng X.L., Liang Z.H., Tang W.W., 2000. Cloning and sequencing of the citrus Huanglongbing pathogen DNA. *Acta Phytopathologica Sinica* **30**: 71-75.

Li X.M., 2004. Recent news on China fruit trade and development strategy. *News of Citrus and Subtropical Fruit* **20**: 10-13.

Li T., Ke C., 2002. Detection of bearing rate of *Liberobacter asiaticum*, in citrus psylla and its host plant *Murraya panciculata* by Nested-PCR. *Acta Phytopathologica Sinica* **29**: 31-35.

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

Planet P., Jagoueix S., Bovè J.M., Garnier M., 1995. Detection and characterization of the Africa citrus greening *Liberbacter* by amplification, cloning and sequencing of the *rpl*KAJL-*rpo*BC operon. *Current Microbiology* **30**: 137-141.

Regmi C., Garnier M., Bové J.M., 1996. Detection of the Asian Huanglongbing (Greening) Liberobacter in Nepal by DNA-DNA Hybridization. In: da Graça J.V., Moreno P., Yokomi R.K. (eds.). *Proceedings of the 13th Conference of the International Organization of Citrus Virologists, Riverside* 1996, 267-270.

Tsushima S., Hasebe A., Komoto Y., Carter J.P., Miyashita K., Yokoyama K., Pickup R.W., 1995. Detection of genetically engineered microorganism in paddy soil using a simple and rapid 'nested' polymerase chain reaction method. *Soil Biology and Biochemistry* **27**: 219-227.

Villechanoux S., Garnier M., Renaudin J., Bovè J.M., 1993. The genome of the non-cultured bacterial-like organism associated with citrus greening disease contains the *nus*G-*rpl*KAJL-*rpo*BC gene cluster and the gene for a bacteriophage DNA polymerase. *Current Microbiology* **26**: 161-166.

Weng S.Z., 1997. Planting technique of special south fruits. Guangdong Academic Press, Guangzhou, China.

Yi G.J., Huo H.Q., Cai C.C., Qiu Y.P., Huang Z.R., Cheng D.C., 1999. DNA isolation procedure for AFLP amplification reaction of Litchi. *Journal of South China Agricultural University* **20**(3): 123-124.

Zhuang S.K., 2003. Yang Cun citrus Huanglongbing and it's managing system. *South China fruits* **32**(5): 17-19.

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