Eur J Plant Pathol (2009) 124:495–503
DOI 10.1007/s10658-009-9436-0

Phylogenetic analysis of the citrus Huanglongbing (HLB) bacterium based on the sequences of 16S rDNA and 16S/23S rDNA intergenic regions among isolates in China

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Received: 15 August 2008 / Accepted: 29 January 2009 / Published online: 14 April 2009

Abstract Phylogenetic analysis of Chinese isolates of the citrus Huanglongbing (HLB) bacterium based on the 16S rDNA and 16S/23S rDNA intergenic regions sequences was carried out. Nine HLB samples collected from different hosts with different symptoms in seven Chinese provinces, were subjected to PCR for amplifying and sequencing the 16S rDNA. The identity level among Chinese isolates was 98.5% to 100% and was the same with the Indian HLB isolate 'Poona' (GenBank accession number: L22532). By contrast, identity values were 97.5% to 97.8% with *Candidatus Liberibacter africanus* strain 'Nelspruit' (L22533), 96.3% to 97.3% with *Ca. L. africanus* subsp. 'Capensis' (AF137368), 95.3% to 96.5% with the *Ca. Liberibacter* sp. 'LSg2' (AY919312), and 94.9% to 96.0% with a strain of *Ca. L. americanus* from Brazil (São Paulo State; AY742824). A phylogenetic tree constructed with 16S rDNA sequences showed that all Chinese isolates belong to *Ca. L. asiaticum*. Analysis of the 16S/23S rDNA intergenic region was conducted on 18 HLB-diseased citrus samples with different symptoms, collected in seven provinces. These isolates showed no obvious variation and had an identity level >99.0% with one another. Sequence analysis of 16S/23S rDNA intergenic region and the relative phylogenetic tree showed that the Chinese isolates are very close to *Ca. L. asiaticus*, and distinct from *Ca. L. africanus* and *Ca. L. americanus*. These results suggest that the Chinese HLB isolates belong to the species *Candidatus Liberibacter asiaticus*. This is the first report on the classification of HLB isolates from China based on molecular investigations.

Keywords Citrus Huanglongbing · 16S rDNA · 16S/23S rDNA · Phylogenetic analysis

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Introduction

Citrus Huanglongbing (HLB), previously known as 'greening', is one of the most destructive diseases of citrus in Asia, south–east Asia, south and east Africa, the Arabian Peninsula (Da Graca 1991; Garnier and Bovè 1993; Garnier and Bové 1996; Bové 2006), and the Americas (Halbert 2005. Pest Alert: Citrus

Greening/Huanglongbing <http://www.doacs.state.X.us/pi/chrp/greening/citrusgreeningalert.html/>).

HLB infects citrus trees of almost all cultivars and causes substantial economic losses to the industry by adversely affecting the crop and shortening the lifespan of infected trees (Bové 2006; Ding et al. 2008). It is estimated that, globally, >60 million trees have already been killed (Das et al. 2007; Halbert and Manjunath 2004), making HLB one of the most serious disorders in many citrus-growing countries, including China (Ding 2006).

The causal agent is a phloem-limited, non-cultured, Gram-negative bacterium (Garnier et al. 1984). 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 (β-operon; Planet et al. 1995; Villechanoux et al. 1993), this bacterium was shown to represent a new genus in the α-subdivision of the *Proteobacteria*, and was assigned the *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 are different species, originally denoted *Candidatus Liberibacter asiaticum* and *Candidatus Liberibacter africanum*, respectively (Jagoueix et al. 1994, 1997), and were later re-named *Ca. L. asiaticus* and *Ca. L. africanus* (Garnier et al. 2000). A third species, *Candidatus Liberibacter americanus*, has recently been reported from Brazil (Coletta-Filho et al. 2005).

The earliest record of HLB disease in China dates back to 1919 in Chaozhou district of Guangdong province (Reinking 1919). Ever since then, HLB has been spreading in the country, so that it now occurs in 11 southern provinces, including Guang Dong, Guang Xi, Zhe Jiang, Fu Jian, Tai Wan, and Hai Nan (Ding et al. 2004, 2005; Deng et al. 2008; Tsai et al. 2008).

Since the extant classification of the HLB bacterium from China as *Ca. L. asiaticus* is not based on molecular evidence, investigations were carried out to determine its taxonomic status and to establish the relationships of Chinese HLB isolates with known Asian, African, and American strains.

Materials and methods

Plant material

Material was collected in different Chinese provinces from Ponkan (*Citrus reticulata*), Wampee (*Clausena lansium*), Shatian pomelo (*C. grandis*) and other hosts showing different symptoms. Samples used in the investigations are listed in Table 1, and the places of collection are shown in Fig. 1. Experimentally infected periwinkle (*Catharanthus roseus*) obtained from Yang Cun Oversea Citrus Research Institute was used as the HLB positive control; negative controls were leaves of healthy trees of the same species and varieties as above grown in an insect-proof greenhouse.

HLB identification by indexing

Field surveys for HLB disease were carried out in September 2003 and April 2004. Buds were collected from citrus trees with HLB-type symptoms, i.e. small-sized leaves with vein yellowing and blotchy mottling or nutritional deficiency-like symptoms. Shoots were side-grafted on Ponkan seedlings (*C. reticulata*) kept in a glasshouse at 30/25°C (day/night) and a photoperiod 14 h light and 10 h darkness, after an initial incubation at 25/20°C for 3 weeks. Weekly observations were made for symptom appearance.

Genomic DNA isolation

Total genomic DNA was extracted according to Ding et al. (2004, 2005). Briefly, 0.3 g of chopped midribs were frozen in liquid nitrogen, and then quickly ground to a fine powder with a mortar and pestle. The powder was collected in 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% ethanol and 95%

Table 1 HLB samples for 16S rDNA and 16S/23S rDNA analysis

Host species and cultivars	Geographic origin	Code name	Symptoms
Ponkan (*C. reticulate*)[a]	Guang Dong	Gd-pg	Yellowing
Luogang sweet orange (*C. sinensis*)	Guang Dong	Gd-ltc	Blotchy mottling
Wampee (*Clausena lansium*)[a]	Guang Dong	Gd-hp	Yellowing
Eureka (*C. limon*)	Guang Dong	Gd-ylk	Nutrition deficiency
Shatang orange (*C. microcarpa*)	Guang Dong	Gd-stj	Yellowing
Tsunokaori (Kiyomi tangor × *C. unshiu*)[a]	Chong Qing	Cq-tk	Blotchy mottling
Qingjian (*C. unshiu* × *C. sinensis*)	Chong Qing	Cq-qj	Yellowing
Shatian pomelo (*C. grandis*)[a]	Guang Xi	Gx-sty	Blotchy mottling
Wenzhou orange (*C. unshiu*)	Guang Xi	Gx-wg	Yellowing
Hongjiang sweet orange(*C. sinensis*)[a]	Guang Xi	Gx-nn	Nutrition deficiency
Hongjiang sweet orange(*C. sinensis*)	Guang Xi	Gx-gl	Blotchy mottling
Beijing lemon (*C. limon*)[a]	Hai Nan	Hn-bjnm	Nutrition deficiency
Newhall navel orange[a]	Fu Jian	Fj-nhe	Yellowing
Wenzhou orange (*C. unshiu*)	Fu Jian	Fj-wg	Yellowing
Nan Gan (*C. nushiu. cv.*)[a]	Hu Nan	Hn-ng	Yellowing
Guanxi orange (*C. grandis. cv.*)	Hu Nan	Hn-gxmy	Yellowing
Gong Chuan (*Miyagawa wase unshu*)[a]	Jiang Xi	Jx-gc	Blotchy mottling
Newhall navel orange (*C. sinensis*)	Jiang Xi	Jx-nhe	Yellowing

[a] Samples used for 16s rDNA analysis. All samples were used for 16S/23S rDNA analysis

Fig. 1 Sites of collection of symptomatic samples

ethanol, air-dried, and resuspended in 100 μl of double distilled water. The recovered DNA was used as template for PCR with *Ca. L. asiaticus*-specific PCR primers for 16S rDNA and 16S/23S rDNA intergenic regions analysis.

PCR amplification of 16S rDNA and 16S/23S rDNA intergenic regions

PCR was performed in a 25 μl volume with the reaction mixtures: 10 μl PCR buffer 2.5 μl, 0.1 mM of each dNTP, 0.4 mM of each primer, one unit of *Taq* DNA polymerase (TaKaRa, Dalian, China), and 10 ng genomic DNA. The primer pair OI1 5′-GCGCGTATGCAATACGAGCGGCA-3′, and OI2c 5′-GCGTCGCGACTTCGCA ACCCAT-3′ (Jagoeix et al. 1994) was used for amplification of the 16S rDNA regions whereas the 16S/23S rDNA intergenic region of each isolate was amplified with primers OI2 (5′-ATGGGTTGCGAAGTCGCGAGGC-3′) and 23S1 (5′-CGCCCTTC TCTCGCGCTTGA-3′; Subandiyah et al. 2000). Cycling parameters were: (1) 16S rDNA, 95°C for 3 min, followed by 35 cycles consisting of denaturation at 95°C for 40 s, annealing at 65°C for 1 min and extension at 72°C for 2 min, and final extension at 72°C for 10 min; (2) 16S/23S rDNA intergenic region, 95°C for 40 s, 55°C for 40 s, and 72°C for 90 s. PCR was carried out in a 96-well block PCR Thermal Cycler (Model PTC-100, MJ Research, USA).

The amplified PCR products were separated by electrophoresis in 1.5% agarose gels (1× TBE buffer) with ethidium bromide staining (0.5 μg ml^{-1}) before and after incubation with the restriction enzyme *Xba*I. The gel was visualised under UV light.

Cloning of DNA and sequence analysis

The expected PCR products were cloned and sequenced (Ding et al. 2005). The sequence analysis started by searching the identity in NCBI Blast, then used the DNAman (Lynnon BioSoft, 5.2. 2.0), Clustal X(EMBL-EBI, 1.81) and DNAstar software (InstallShield Software Corporation, InstallShield, 5.00.221.0) for multiplex alignment and phylogenetic tree construction. Bootstrapping (10,000 replications) was performed to estimate stability and support for the inferred clusters.

Results

Graft transmission tests

Of the 30 samples collected in seven Chinese provinces (Fig. 1), 18 induced severe vein yellowing, leaf mottle and nutritional-like deficiency symptoms in Ponkan within 6 months from grafting (Fig. 2), thus were retained as affected by HLB.

PCR amplification of 16S rDNA and 16S/23S rDNA intergenic regions

A 1,160 bp target sequence of 16S rDNA and a 900 bp target sequence of the 16S/23S rDNA intergenic region were amplified. Strong positive results were obtained from HLB-diseased leaves, including Gd-pg, Gd-hp, Gx-sty, Gx-nn, Hn-bjnm, Fj-nhe, Cq-tk, Hn-ng, Cq-qj, Jx-gc and Gd-stj. No amplification was obtained from the non-inoculated Ponkan as well as from Luogang sweet orange from Guang Dong province and Hongjiang sweet orange from Hai Nan province which showed symptoms similar to a nutritional deficiency, and twelve other plants did not develop greening symptoms after grafting (data not shown).

As reported by Jagoeix et al. (1996), the restriction enzyme *Xba*I hydrolyses the 16S rDNA of *Ca. L. africanus* into three fragments (520, 506 and 130 bp) and that of *Ca. L. asiaticus* into two fragments (640 and 520 bp). When amplified DNAs of Gd-pg (lane 1), Gd-hp (lane 2), Gx-sty (lane 3), Gx-nn (lane 4), Hn-bjnm (lane 5), Fj-nhe (lane 6), Cq-tk (lane 7), Hn-ng (lane 8), and Jx-gc (lane 9) were submitted to *Xba*I digestion and analysed on 2% agarose gels, the expected restriction pattern was obtained (Fig. 3)

According to a previous report (Jagoeix et al. 1997; Subandiyah et al. 2000), the amplification of the intergenic region between 16S and 23S rDNA resulted in a fragment of about 900 bp. In this study, in relation to the 16S/23S rDNA intergenic region, primers OI2 and 23S1 amplified products of a similar size (about 900 bp) from bacterial isolates coming from Gd-pg, Gd-hp, Gx-sty, Gx-nn, Hn-bjnm, Fj-nhe, Cq-tk, Hn-ng, Fj-wg, Jx-gc, Gd-stj, Hn-gxmy Gd-ltc, Gd-ylk, Cq-qj, Gx-wg, Gx-gl and Jx-nhe. However, no amplificon products were obtained form any negative controls including the

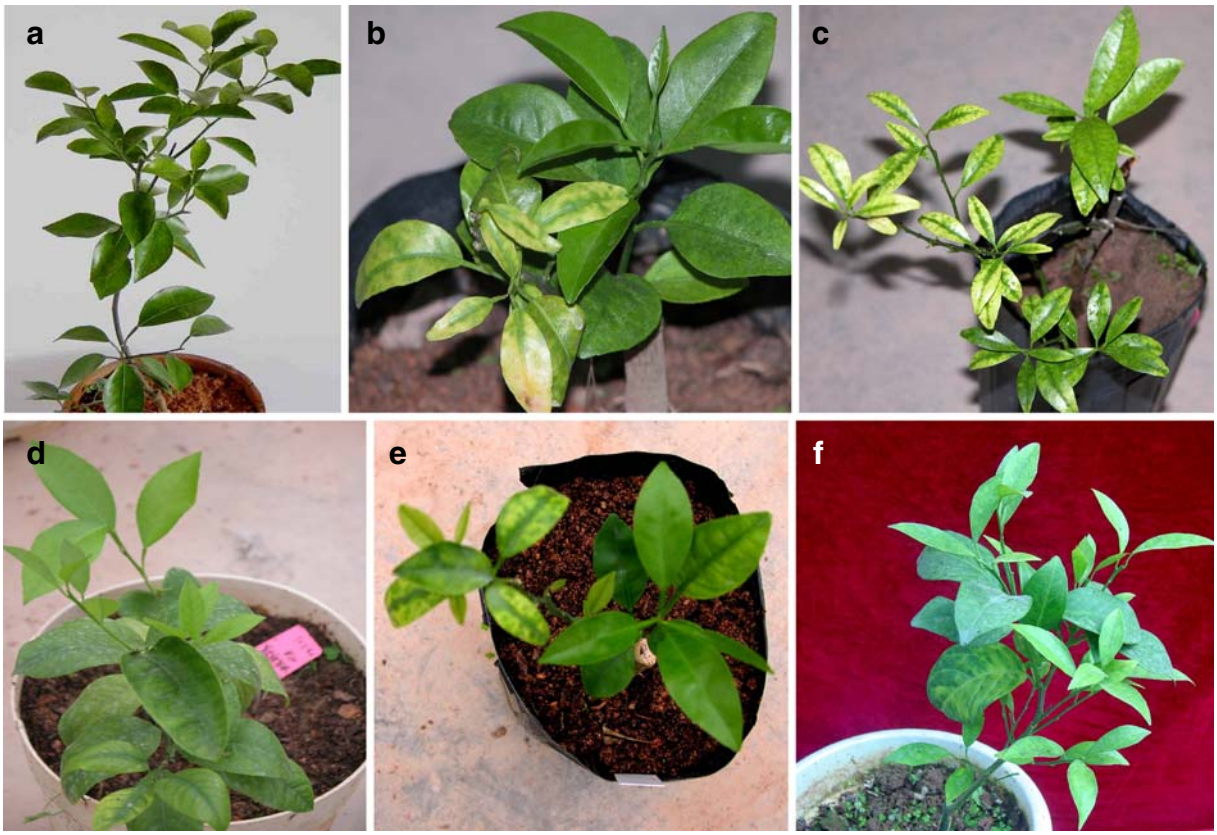

Fig. 2 Symptoms of HLB disease on indicator plants (22 weeks after grafting): **a** Healthy ponkan. **b** Small-sized yellow leaves caused by Gd-ltc. **c** Yellow leaves with narrow blades typically caused by Cq-tk. **d** Symptoms resembling nutritional deficiency caused by Hn-bjnm. **e** Leaf yellowing caused by Cq-qj. **f** Blotchy mottling caused by Jx-gc

non-inoculated Ponkan, Luogang sweet orange from Guang Dong province, Hongjiang sweet orange from Hai Nan province and the other 12 plants, which did not develop HLB symptoms after grafting. These results were consistent with those of the 16S rDNA amplification.

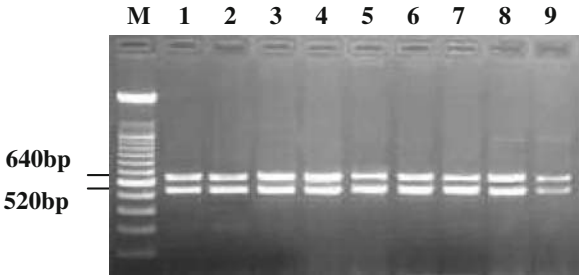

Fig. 3 Agarose gel electrophoresis of 16S rDNA digested with restriction enzyme *Xba*I. M,100 bp DNA ladder marker VII (SD015-2, Dingguo); *lane 1*, Gd-pg; *lane 2*, Gd-hp; *lane 3*, Gx-sty; *lane 4*, Gx-nn; *lane 5*, Hn-bjnm; *lane 6*, Fj-nhe; *lane 7*, Cq-tk; *lane 8*, Hn-ng; *lane 9*, Jx-gcF. Blotchy mottling caused by Jx-gc

16S rDNA sequence analysis and phylogenetic tree construction

Nine 16S rDNA amplified fragments of three different clones were sequenced in both orientations. The fragment amplified from 16S rDNA was 1,167–1,168 bp in size (GenBank accession numbers DQ431997–DQ432005). When these sequences were compared with those of other liberibacters from the database, a 98.5% to 100% identity was found with *Ca. L. asiaticus* strain 'Poona' (GenBank accession number L22532); 97.5% to 97.8% and 96.3% to 97.3% identity with *Ca. L. africanus* strain 'Nelspruit' (L22533) and *Ca. L. africanus* subsp. 'Capensis' (AF137368), respectively; 95.3 to 96.5% identity with *Ca. Liberibacter* sp. strain 'LSg2' (AY919312); and 94.9% to 96.0% identity with *Ca. L. americanus* São

Paulo strain (AY742824). The nine Chinese HLB isolates were virtually identical (98.5–100%) to one another (Table 2).

A phylogenic tree constructed with 16S rDNA sequences (Fig. 4) showed that the evolutionary distance among our HLB isolates and those previously sequenced in China, i.e. Guangdong (DQ303210, DQ157273), Guizhou (DQ157275) and Guangxi (DQ157274) were close to the Asian strain 'Poona', but not to the African strains 'Capensis' and 'Nelspruit'. The genetic distance with the American strain was wider than with African strains 'Capensis' and 'Nelspruit'. However, all HLB isolates including those from Asia, Africa and America were classified to α-subdivision as defined by Murray and Schleifer (1994) distinct from those of β- and γ-subdivisions. This was retained as evidence that the HLB isolates from China also belong to α-subdivision.

16S/23S rDNA sequence analysis and phylogenetic tree construction

All 16S/23S rDNA amplicons from the 18 citrus isolates of the HLB bacterium consisted of a single band, indicating the intergenic spacer region of the citrus HLB bacterium may have only one configuration. The amplified fragments of three random clones of 16S/23S rDNA were sequenced in both orientations. The determined sequence was 912 bp, including the 16S rDNA terminal region and the 598 bp intergenic spacer region (GenBank accession numbers DQ462240–DQ462257).

The average identity level in the entire 16S/23S intergenic regions of the 18 isolates was 99.7%. Two putative *tRNA* genes separated by 11 nucleotides, $tRNA^{Ile}$ (nucleotides 186 to 262) and $tRNA^{Ala}$ (nucleotides 274 to 349), were identified in the intergenic region of all isolates. These sequences had 99.7–100% identity with those of the Asian strain 'Poona' (U61359) and the Okinawa island strain 'OKS7' (AB019793), 99.8-100% identity with the Florida strain '12166' (EU265647), 99.7–100% identity with the China Taiwan strain 'Kumquat 1', but shared only 74.7–74.9% identity with the African strain 'Nelspruit' (U61360), and 76.6–77.1% with *Ca L. americanus* strain São Paulo (AY859542).

The phylogenic tree constructed with the 16S/23S intergenic regions sequences (Fig. 5) was similar to that constructed with 16S rDNA sequences. All HLB isolates from China grouped in the same cluster together with the Asian strain 'Poona' (U61359) and Okinawa island strain 'OKS7' (AB019793). The African strain 'Nelspruit' (U61360) and the *Ca. L. americanus* strain (AY859542) were both at located at different positions. Thus, it was confirmed that the HLB Chinese isolates belong in the α-subdivision together the African and the South American strains (Brazil) from which, however, they are phylogenetically distinct.

Discussion

Diagnosis of HLB is traditionally based on the reaction of indicator plants. In the present study, of 30 citrus sources collected in seven Chinese provinces, 18 induced severe vein yellowing, leaf mottle and nutritional-like deficiency symptoms in Ponkan, within 6 months from grafting. The lack of reaction from the remaining twelve sources may be due either to the erratic distribution of the HLB bacterium in the sampled host, or to the fact that the sampled trees were affected

Table 2 Identity and distance matrix of 16SrDNA sequences among nine different HLB isolates

	Cq-tk	Gx-nn	Fj-nhe	Gd-hp	Gd-pg	Hn-ng	Gx-sty	Jx-gc	Hn-bjnm
Cq-tk	***	0.014	0.015	0.015	0.015	0.015	0.015	0.014	0.014
Gx-nn	100.0%	***	0.005	0.005	0.005	0.005	0.005	0.003	0.003
Fj-nhe	99.5%	99.5%	***	0.007	0.007	0.007	0.007	0.005	0.005
Gd-hp	99.5%	99.5%	99.3%	***	0.000	0.000	0.007	0.005	0.005
Gd-pg	99.5%	99.5%	99.3%	100.0%	***	0.000	0.007	0.005	0.005
Hn-ng	99.5%	99.5%	99.3%	100.0%	100.0%	***	0.007	0.005	0.005
Gx-sty	99.5%	99.5%	99.3%	99.3%	99.3%	99.3%	***	0.005	0.005
Jx-gc	99.7%	99.7%	99.5%	99.5%	99.5%	99.5%	99.5%	***	0.000
Hn-bjnm	98.6%	98.6%	98.5%	98.5%	98.5%	98.5%	98.5%	98.6%	***

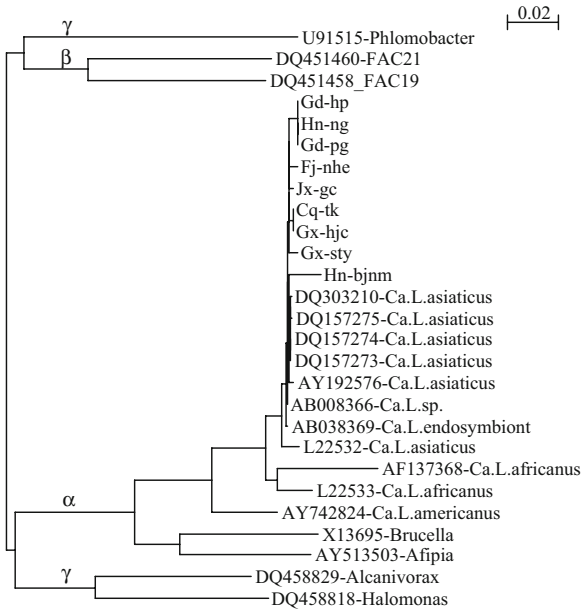

Fig. 4 Phylogenetic tree constructed using the neighbour-joining method, based on 16S rDNA sequence data for Chinese HLB bacterium isolates together with other representative isolates from GenBank (bootstrapping is 10,000). *Bar*, 0.02 sequence divergence

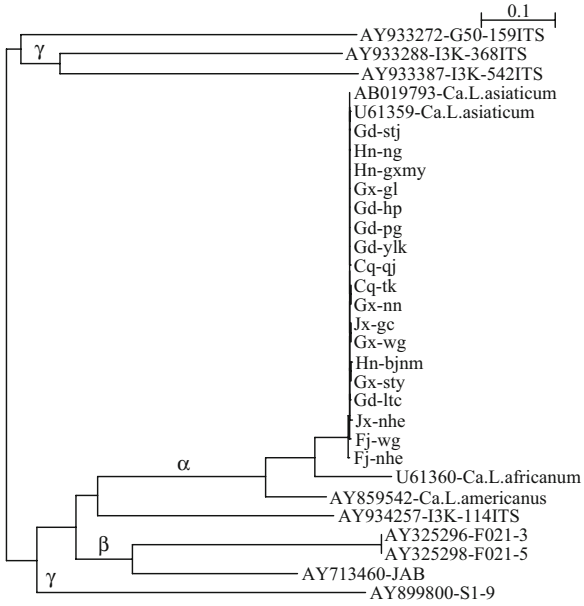

Fig. 5 Phylogenetic tree constructed using the neighbour-joining method, based on 16S/23S rDNA sequence data for Chinese HLB bacterium isolates together with other representative isolates from GenBank (bootstrapping is 10,000). *Bar*, 0.1 sequence divergence

by other diseases inducing symptoms somewhat similar to HLB (e.g. stubborn disease or tristeza) or to abiotic disorders (e.g. zinc deficiency). 16S rDNA sequences are useful for establishing phylogenetic relationships of bacteria at the intra- and intergeneric levels (Stackebrandt et al. 1992; Woese 1987); however, their resolution is not always sufficient to differentiate between closely related species (Ash et al. 1991; Fox et al. 1992; Vandamme et al. 1996). By contrast, sequence variability in the 16S/23S intergenic region is useful for differentiating strains among and within species (Leblond-Bourget et al. 1996).

Taking the above into account, we have used the 16S rDNA and 16S/23S intergenic regions to assess the extent of diversity among HLB isolates from different Chinese regions. Results showed that 16S rDNA sequences were very conserved in most samples, as nine representative isolates yielded single 1,160 bp bands 98.5% to 100% identical to one another. The restriction enzyme *Xba*I, hydrolysed the 16S rDNA amplicon into two fragments (640 and 520 bp), consistent with a previous report (Jagoueix et al. 1996). Of the nine HLB isolates, Gd-pg, Hn-ng and Gd-hp had identical sequences, the same as for Gx-nn, Cq-tk. Some nucleotide substitutions (18 sites) were present in the isolates Gx-sty, Fj-nhe, Hn-bjnm, and Jx-wg. Thirteen nucleotide changes were detected in the Hn-bjnm sequence, most of which consisted of base substitutions (five transitions and five transversions).

Results of 16S/23S rDNA intergenic region analysis of the 18 HLB isolates, showed that although these were collected in different areas, from different hosts with different symptoms, their DNA sequence was highly conserved, with identity ranging from 99.7% to 100%. In particular, isolates Gd-hp, Gd-ylk, Gd-stj, Gx-wg, Gx-gl, Hn-ng, and Hn-gxmy had identical sequences, whereas a small number of nucleotide substitutions (14 sites) was detected in isolates Gd-ltc,

Table 3 16S/23S intergenic spacer region sequence difference between the Chinese (GD-LG-LTC) and Indian (Poona) isolates of the HLB bacterium

	Nucleotide positions						
Isolate	178	219	228	287	318	338	534
Gd-ltc	–	C	G	C	G	–	G
Poona	T	–	–	T	–	C	–

Cq-qj, Gx-sty, Gx-nn, Hn-bjnm, Fj-nhe, Fj-wg, Cq-tk, Jx-gc and Gx-wg. These sequences are very similar to the reported Indian 'Poona' (99.7–100%). The sequence differences in the 16S/23S intergenic region between the HLB isolate from China (Gd-ltc) and that from India (Poona) are presented in Table 3.

Chinese HLB isolates are considered to be of the Asian rather than the African type based on tolerance to high temperature and the restriction *Xba*I pattern of the PCR fragment of 16S rDNA. As stated previously, sequences of the amplified 16S rDNA and 16S/23S rDNA intergenic region are identical among Chinese isolates and, in turn, very similar to those reported for the Indian (Jagoueix et al. 1994, 1997), Japanese (Subandiyah et al. 2000), Thai and Nepalese (Nakashima et al. 1996, 1998; Ohtsu et al. 1998) isolates, but distinct from the sequence of the African strain (Jagoueix et al. 1997).

Thus, it seems plausible to conclude that HLB isolates from China, North America (Florida), India, Japan, Thailand, and Nepal constitute one strain which, based on differences in the 16S rDNA and the 16S/23S intergenic spacer region sequences, is distinct from the African HLB bacterium.

Acknowledgements This study was supported by National Natural Science Foundation of China (no. 30700550), Doctoral Fund-Youth Scholars of Ministry of Education of China (no. 20070504079), China Ministry of Education (no. IRT0548), and Key Plan of Hubei province (no. 2006AA203B05). We especially thank Prof. G.P. Martelli, University of Bari, Italy for revising the manuscript.

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