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# Plant Pathology



An International Journal edited by  
the British Society for Plant Pathology  
**Senior Editor Matt Dickinson**



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# Plant Pathology

An international journal edited by the British Society for Plant Pathology

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# Occurrence of prophage and historical perspectives associated with the dissemination of huanglongbing in mainland China

S. Fu<sup>a†</sup>, Z. Bai<sup>b†</sup>, H. Su<sup>c</sup>, J. Liu<sup>a</sup>, J. S. Hartung<sup>d</sup>, C. Zhou<sup>a</sup> and X. Wang<sup>a\*</sup>

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Huanglongbing (HLB), associated with a non-culturable bacterium ‘*Candidatus Liberibacter asiaticus*’ (CLAs), is a highly destructive citrus disease with a long but poorly documented history in China. No effective treatment for HLB is available. The identification of new prophages in abundant CLAs genomic sequence data provides new insights into both the diversity of CLAs strains and HLB management. In this study, CLAs populations from nine provinces were surveyed for the presence of prophage. Two major prophage typing groups (PTGs) were discovered to be associated with two different altitude regions: strains of CLAs in PTG1 from high altitude regions (HAR) mainly contained prophage Type 1 only or Types 1 and 3, whereas strains of CLAs in PTG2 from low altitude regions (LAR) mainly contained prophage Type 2. The discovery of these CLAs population patterns provides evidence for independent origins of HLB in HAR and LAR. Guangdong province is the generally recognized domestic region of origin for HLB and is primarily responsible for the dissemination of HLB in LAR through transport of seedlings. Both Yunnan and Sichuan provinces are the probable regions of origin for HLB in HAR. PTG2 was further divided into two subgroups: PTG2-1, found in Guangdong, Fujian and Guangxi and PTG2-2, found in Jiangxi, Zhejiang and Hunan. These regions and prophage types are correlated with early and late introductions of HLB in LAR. These molecular analyses were supported by studying the history of the dissemination of HLB in historical documents.

**Keywords:** bacteriophage, ‘*Ca. Liberibacter asiaticus*’, epidemiology, huanglongbing (HLB)

## Introduction

Huanglongbing (HLB), also known as citrus greening, is the most destructive disease of citrus and is distributed in more than 40 countries (Bové, 2006). HLB is associated with three nonculturable and phloem-restricted  $\alpha$ -proteobacteria species, ‘*Candidatus Liberibacter asiaticus*’ (CLAs), ‘*Ca. Liberibacter africanus*’ (CLaf; Jagoueix *et al.*, 1994) and ‘*Ca. Liberibacter americanus*’ (CLam; Teixeira *et al.*, 2005). CLAs and CLam are transmitted by the Asiatic citrus psyllid *Diaphorina citri* and CLaf is transmitted by the African citrus psyllid *Trioza erythrae* (Bové, 2006). CLAs is the only species found and verified in both Asia and North America, while both CLam and CLAs are found in South America, although CLAs has outcompeted CLam to become the dominant species (Lopes *et al.*, 2009).

Brazil and the USA are two major citrus producers. Both countries have been damaged by HLB since the

mid-2000s; the economic losses caused by HLB have increased significantly in recent years and have attracted great research interest supported by government and industry (da Graça *et al.*, 2016). HLB was present in mainland China more than a century ago and was first described in Chaoshan, Guangdong province in 1919 (Reinking, 1919). Although vigorous efforts have been undertaken to control HLB over the past century, it has spread to 11 of 20 citrus-growing areas in China. Biological research on CLAs is extremely challenging due to lack of *in vitro* cultures. Historically, the 16S rDNA and  $\beta$ -operon regions of the ribosomal protein genes have been used to detect the presence of CLAs (Deng *et al.*, 2008), but they cannot be used to differentiate distinct strains of CLAs within countries. An approach that used *omp*-based PCR restriction fragment length polymorphism (RFLP) was developed that detected a single CLAs strain from Yunnan and a unique RFLP profile among strains from Guangxi (Bastianel *et al.*, 2005). With the availability of CLAs genomes and the discovery of Type 1 and Type 2 prophage sequences integrated into bacterial chromosomes (Zhang *et al.*, 2011), prophage regions

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are widely used to improve the resolution of population diversity studies. For example, prophage terminase genes were used to differentiate CLas strains from Guangdong and Yunnan (Liu *et al.*, 2011). DNA mosaicism and hypersequence variation were identified and successfully used to differentiate CLas strains from Yunnan and Guangdong following PCR of several prophage genes and the documentation of dominant electrophoretic profiles (E-types; Wang *et al.*, 2012). A Type 3 prophage was discovered recently (Zheng *et al.*, 2017) and employed for diversity analysis of CLas strains in California and southern China (Dai *et al.*, 2019; Li *et al.*, 2019).

Studies of CLas diversity demonstrated a tendency for regional populations in China and have been focused in Guangdong and Yunnan (Deng *et al.*, 2008; Liu *et al.*, 2011), provinces with significantly different geography, providing an interesting model for the analysis of diversity in CLas populations. Recently, the history and development of HLB in Guangdong was reviewed and HLB was considered to be endogenous (Zheng *et al.*, 2018). However, the focus on Guangdong did not provide information about the epidemiology and distribution of HLB in the rest of mainland China. In order to provide a better understanding of the diversity of CLas strains in mainland China, the present investigation used CLas strains collected from nine provinces and differentiated them by prophage typing. The different prophage typing groups of CLas strains are discussed in the context of geographical origin and chronological dispersal of HLB based on historical documents.

## Materials and methods

### Collection of HLB samples and prophage typing

Samples ( $n = 368$ ) with suspect HLB symptoms, including yellowing, zinc deficiency and blotchy mottle, were collected from nine provinces between 2014 and 2019. Samples were taken from trees, put in zipper bags with wet paper towels and shipped to the Citrus Research Institute of Southwest University, Chongqing, China. For each tree, a DNA sample was extracted from the midribs of fresh leaves using the CTAB method (Murray & Thompson, 1980) and designated as a CLas strain as described below. The presence of CLas was preliminarily verified by PCR with the primer set OI1/OIc2 (Jagoueix *et al.*, 1996). Subsequently, six primer sets were used to detect prophages, including T1-2F/T1-2R and T1-3F/T1-3R specific to prophage Type 1, T2-2F/T2-2R and T2-3F/T2-3R specific to prophage Type 2 and 891-1F/891-1R and 891-2F/891-2R specific for prophage Type 3 (Zheng *et al.*, 2017). CLas strains were then classified into eight prophage-based groups: Type 1, Type 1 + Type 2, Type 1 + Type 3, Type 1 + Type 2 + Type 3, Type 2, Type 2 + Type 3, Type 3 and no prophage. In order to eliminate stochastic errors due to sample size and sampling locations, the sample size was enlarged by adding a set of published data (Li *et al.*, 2019). Samples from seven different citrus varieties/species, including *Citrus sinensis*, *C. grandis*, *C. reticulata*, *C. limon*, *C. unshiu*, *C. aurantium* and *C. tangerina*, were collected at different times of year. CLas-infected samples from individual provinces were obtained from their most widely planted cultivars. All primers used in this study are described in Table S1.

### Analysis of CLas populations

A pairwise matrix of genetic distance and genetic identity for CLas strains was generated with POPGENE v. 3.2 (<https://sites.ualberta.ca/~fyeh/index.html>) using Nei's unbiased measure and Nei's (1973) gene diversity ( $H$ -value) was used for CLas populations in association with prophages and individual provinces. The structure of CLas populations was determined with dendrograms built with MEGA 7.0 using the unweighted paired group method with arithmetic mean (UPGMA) with the imported genetic distance matrix. Analysis procedures were as detailed previously (Li *et al.*, 2019).

### Collection of historical references and modelling dispersal of HLB

HLB reports, both published and retained locally but unpublished, were collected from the plant protection departments and citrus research units of nine provinces where HLB occurred in mainland China from 2009 to 2018. Personal notes of primary field investigations were donated by Professors X. Y. Zhao of the Citrus Research Institute, Chinese Academy of Agricultural Sciences, X. L. Zhao from Guangxi Academy of Specialty Crops, J. Q. Li from Guizhou Fruit Science Research Institute, C. X. Chen from Ganzhou Fruit Tree Protection Station in Jiangxi province and G. Q. Chen from Zhejiang Citrus Research Institute. With the aid of these documents, the historical epidemiology of HLB was reconstructed chronologically and mapped to exact locations with ArcGIS v. 10.2 software (<http://www.esri.com/arcgis/about-arcgis>).

## Results

### Detection of prophages by PCR

A total of 765 CLas-infected citrus DNA samples collected from nine provinces (Fig. 1) were used to assess population diversity of CLas by detection of the three known prophages. All combinations of prophage were detected except Type 2 + Type 3. A small portion of CLas strains (32/765, 4%) did not carry any prophage. The geographic locations where HLB occurred were grouped into low altitude regions (LAR) and high altitude regions (HAR) based on the geography of mainland China. HAR include provinces Yunnan, Sichuan and Guizhou and LAR were mainly the provinces of Guangdong, Fujian, Guangxi, Jiangxi, Zhejiang and Hunan. The incidence of prophage typing was most abundant in Yunnan and Guangdong provinces.

On average, CLas strains from HAR most frequently contained prophage Type 1 (110/281, 39%) or Type1 + Type 3 (93/281, 33%) (Table 1). Type 1 and Type 3 prophages were found in similar proportions in CLas strains from Yunnan, while CLas populations from Sichuan and Guizhou most frequently contained prophage Type 1 (55/75, 73%) and Type 1 + Type 2 (8/16, 50%), respectively. CLas strains from LAR contained primarily prophage Type 2, particularly Guangdong, Fujian and Guangxi (>75%), followed by Jiangxi, Zhejiang and Hunan (>55%) (Table 1).

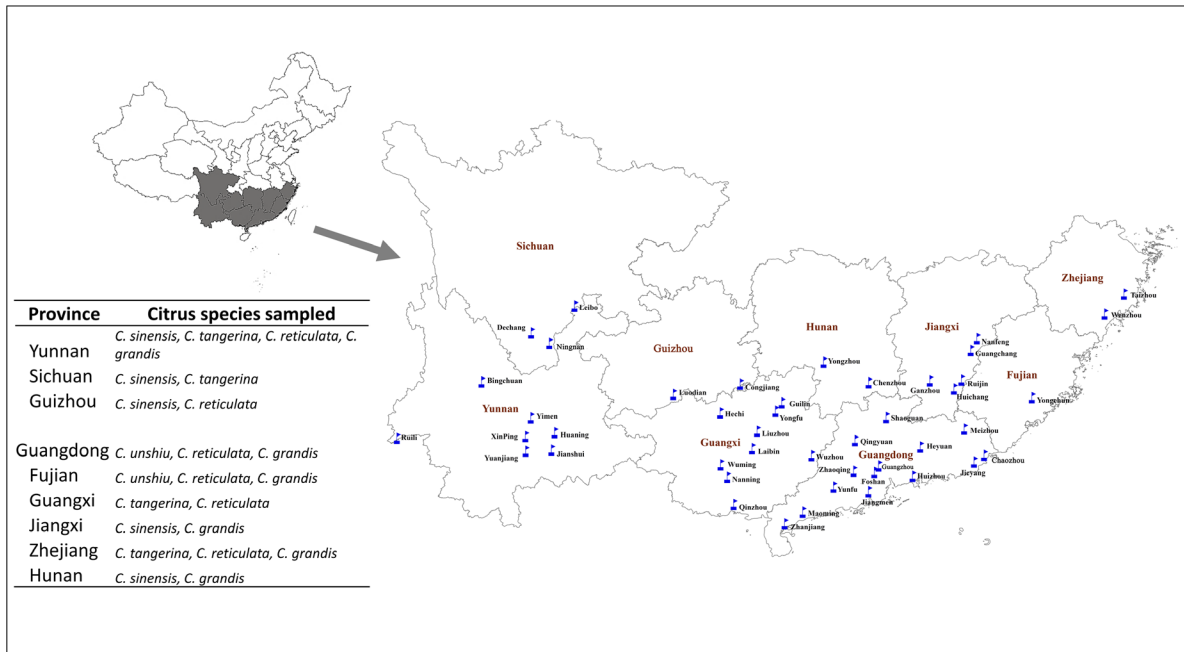


Figure 1 Geographical origins of 'Candidatus *Liberibacter asiaticus*' strains and corresponding citrus species sampled from nine provinces in mainland China.

### Diversity of CLAs populations

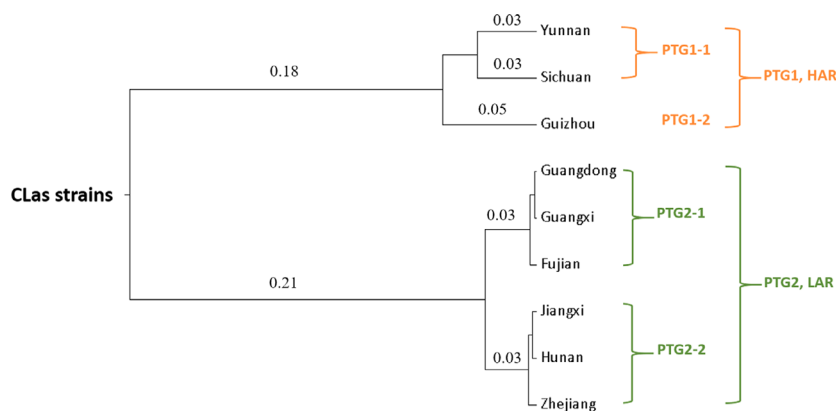
A pairwise population matrix of genetic distance and genetic identity was created for CLAs populations from individual provinces (Table S2). By detection of Type 1, Type 2 and Type 3 prophages (Zheng *et al.*, 2017), CLAs populations from nine provinces were separated into two major groups, prophage typing group 1 (PTG1) from HAR and prophage typing group 2 (PTG2) from LAR, and both major groups were further divided into two subgroups (Fig. 2). CLAs strains from

Yunnan and Sichuan formed subgroup PTG1-1 and CLAs strains from Guizhou formed an independent subgroup, PTG1-2. CLAs strains from Guangdong, Guangxi and Fujian formed subgroup PTG2-1 and CLAs strains from Jiangxi, Hunan and Zhejiang formed subgroup PTG2-2. The *H*-values of genetic diversity for CLAs populations from HAR of Sichuan and LAR of Guangdong, Fujian and Guangxi were relatively low, whereas the *H*-values of HAR of Yunnan and LAR of Jiangxi, Zhejiang and Hunan were relatively high (Table 2).

Table 1 PCR-based detection of three prophage types from citrus samples affected by huanglongbing, collected from nine provinces in China.

	Number of CLas samples with prophage combination (frequency, %)								
Geographical origin	T1	T1 + T2	T1 + T3	T1 + T2 + T3	T2	T2 + T3	T3	None	Total
HAR									
Yunnan	52 (27.4)	5 (2.6)	71 (37.4)	11 (5.8)	35 (18.4)	0 (0)	10 (5.3)	6 (3.2)	190
Sichuan	55 (73.3)	0 (0)	18 (24.0)	0 (0)	1 (1.3)	0 (0)	0 (0)	1 (1.3)	75
Guizhou	3 (18.8)	8 (50.0)	4 (25.0)	0 (0)	1 (6.3)	0 (0)	0 (0)	0 (0)	16
Subtotal	110 (39.1)	13 (4.6)	93 (33.1)	11 (3.9)	37 (13.2)	0 (0)	10 (3.6)	7 (2.5)	281
LAR									
Guangdong	2 (1.2)	10 (6.0)	14 (8.4)	5 (3.0)	129 (77.2)	0 (0)	1 (0.6)	6 (3.6)	167
Fujian	0 (0)	0 (0)	1 (2.6)	0 (0)	33 (84.6)	0 (0)	0 (0)	5 (12.8)	39
Guangxi	0 (0)	0 (0)	11 (8.8)	3 (2.4)	103 (82.4)	0 (0)	2 (1.6)	6 (4.8)	125
Jiangxi	0 (0)	1 (3.7)	3 (11.1)	3 (11.1)	15 (55.6)	0 (0)	2 (7.4)	3 (11.1)	27
Zhejiang	0 (0)	0 (0)	20 (28.2)	1 (1.4)	41 (57.7)	0 (0)	9 (12.7)	0 (0)	71
Hunan	5 (9.1)	0 (0)	13 (23.6)	0 (0)	31 (56.4)	0 (0)	1 (1.8)	5 (9.1)	55
Subtotal	7 (1.4)	11 (2.3)	62 (12.8)	12 (2.5)	352 (72.7)	0 (0)	15 (3.1)	25 (5.2)	484
Total	117 (15.3)	24 (3.1)	155 (20.3)	23 (3.0)	389 (50.8)	0 (0)	25 (3.3)	32 (4.2)	765

T1, prophage Type 1; T2, prophage Type 2; T3, prophage Type 3; HAR, high altitude regions; LAR, low altitude regions.



**Figure 2** The classification of '*Candidatus Liberibacter asiaticus*' populations from nine provinces in mainland China by detection of the presence of prophages. The dendrogram was built using the unweighted paired group method with arithmetic mean (UPGMA) and figures represent genetic distance between strains. PTG, prophage typing group; HAR, high altitude regions; LAR, low altitude regions.

### Historical overview of HLB occurrence in mainland China

Eleven of 20 citrus-growing regions have suffered from HLB in China (Fig. 3). Taiwan and Hainan were excluded from this study due to a lack of historical documentation from the two regions and because citrus is not planted on a large scale in Hainan, which was governed by Guangdong before 1988. Figure 3 shows the history of HLB in mainland China, which can be divided into two periods: HLB was commonly found only in Guangdong, Fujian and Guangxi before the 1970s, after which HLB was observed in Jiangxi, Zhejiang, Hunan, Yunnan, Sichuan and Guizhou. The year and location of the first reports of HLB in individual provinces are indicated with asterisks. Arrows represent the transport of diseased seedlings from Guangdong to Minhou of Fujian in 1938 (Lin, 1963), to Yulin of Guangxi in 1933 (Huang, 1943), to Xingguo of Jiangxi in 1978 (Ganzhou Citrus Huanglongbing Research Group, 1979, Investigation report on the occurrence and distribution of citrus huanglongbing, unpublished), to Pingyang of Zhejiang in 1981 (Z. T. Chen, unpublished report on preliminary

discovery of citrus huanglongbing distribution on the northern margin in China in 1982) and to Yizhang of Hunan in 1981 (Jiang & Qiu, 1982). In Figure 4 and Figures S1–S3, the dynamic spread of HLB and psyllids in mainland China is visualized in greater detail, with different colours for different decades. HLB is distributed throughout Guangdong, Fujian and Guangxi (Fig. S1a–c), whereas it is restricted to a small area in the south of Jiangxi, widespread in southeast Zhejiang (Meng, 2005), has a scattered distribution in Hunan (Fig. S1d–f; Li *et al.*, 1992) and Yunnan, and occurred in small areas in the south of both Sichuan and Guizhou (Fig. S2). The distribution models of psyllids were nearly consistent with the occurrence of HLB in Guangxi, Jiangxi, Hunan, Zhejiang, Sichuan and Guizhou (Fig. S3).

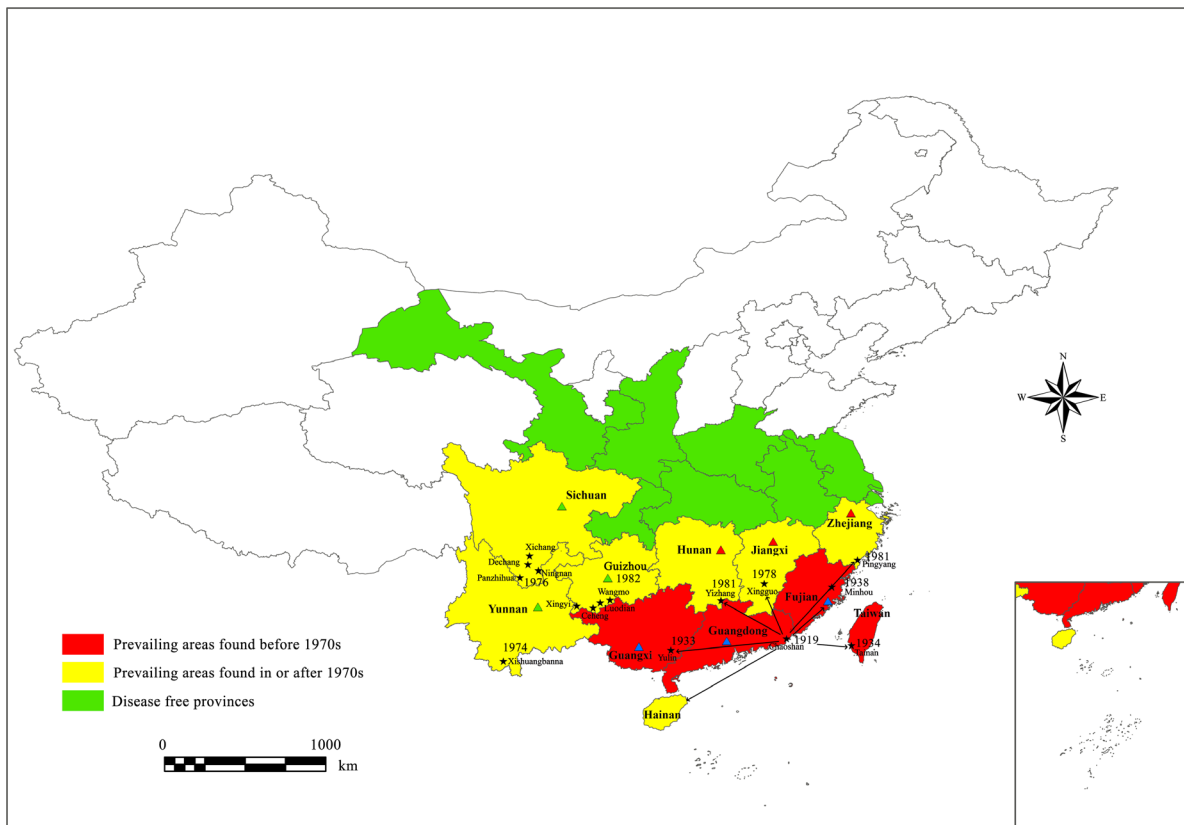
### Discussion

By the detection of three known prophages, two major PTGs were identified in mainland China: PTG1 of HAR and PTG2 of LAR. The remarkable distinction between PTG1 and PTG2 indicates that the two regions might have independent original infections. Interestingly, similar suggestions were made based on phylogenetic studies of Asian citrus psyllids (ACP) and its primary endosymbiont ('*Ca. Carsonella ruddii*'; Wu *et al.*, 2018b), as well as CLas identified with different markers (Liu *et al.*, 2011; Wang *et al.*, 2012). This demonstrates a strong association and co-evolution of CLas with prophages, ACP and '*Ca. Carsonella ruddii*'. The data also demonstrate multiple origins and the dissemination of HLB in mainland China through at least two distinct routes (Wu *et al.*, 2018b). Guangdong was the first province reported with HLB in mainland China in 1919 (Reinking, 1919). Since then, HLB has been reported by Tu (1932), He (1937) and Huang (1943). Recently, researchers speculated that HLB was introduced either from outside China, with Hong Kong and Macau as active foreign trading ports, or was endogenous (Zheng *et al.*, 2018). The wide dissemination of HLB within Guangdong was partially due to propagation of trees by grafting of scions from inadequately screened parental trees, as well as being the result of poor management of

**Table 2** Nei's gene diversity (*H*) of '*Candidatus Liberibacter asiaticus*' populations within provinces of China.

Geographical origin	<i>H</i> -value
<b>HAR</b>	
Yunnan	0.4283
Sichuan	0.1477
Guizhou	0.3281
Region average	0.3014
<b>LAR</b>	
Guangdong	0.2502
Fujian	0.1317
Guangxi	0.2266
Jiangxi	0.4060
Zhejiang	0.4626
Hunan	0.4372
Region average	0.3191
Overall average	0.3102

HAR, high altitude regions; LAR, low altitude regions.



**Figure 3** History of the first reported locations of Huanglongbing (HLB) in China. The maps are divided into provinces and are coloured according to the prevalence or absence of HLB before or after the 1970s. Coloured triangles represent nine provinces from where '*Candidatus Liberibacter asiaticus*' strains were sampled in this study and different prophage typing groups (PTGs): green, PTG1; blue, PTG2-1; red, PTG2-2. Asterisks with years represent the locations and year that HLB was first reported in different provinces. Arrows indicate records of seedling transportation from Guangdong.

abandoned orchards and psyllids (Zheng *et al.*, 2018). In the present study, Guangdong is considered to be the province of origin for HLB in LAR, based on prophage typing and traceable historical documents.

It is interesting to note the detection of subgroups PTG2-1 and PTG2-2 within PTG2, which assisted the understanding of HLB epidemiology in southeastern China, is consistent with the early occurrence of HLB in Guangdong, Fujian and Guangxi (PTG2-1) and its later occurrence in Jiangxi, Hunan and Zhejiang (PTG2-2). In the present study, 1970 was considered as the point of transition because before this time, HLB was found only in Guangdong, Fujian and Guangxi and subsequently, the disease spread rapidly into other provinces. The wide dissemination of HLB in LAR was strongly associated with the intensive transportation of seedlings from Guangdong to Fujian (Lin, 1963), Guangxi (Huang, 1943), Jiangxi (Huang, 1992a), Zhejiang (Z. T. Chen, unpublished report on preliminary discovery of citrus Huanglongbing distribution on the northern margin in China in 1982) and Hunan (Jiang & Qiu, 1982). Following the first incursions of HLB into Fujian and Guangxi through introduction of seedlings, widespread infections within the provinces were primarily disseminated by psyllids (Zhao

*et al.*, 1979; Zhou *et al.*, 1988; Fujian Agricultural Science Experimental Station, 1974, unpublished). Jiangxi, Zhejiang and Hunan are relatively newly invaded areas and the CLAs populations there have greater prophage-associated gene diversity. Greater genetic diversity was also detected in ACP in these provinces (Meng *et al.*, 2018). This strongly suggests that CLAs in these areas has experienced a series of recent population expansion events through introductions of infected seedlings from multiple diseased provinces (Huang, 1992b; Ganzhou Citrus Huanglongbing Research Group, unpublished investigation report on occurrence and distribution of citrus Huanglongbing in 1979; Institute of Subtropical Crops, Zhejiang Academy of Sciences, unpublished investigation report of citrus Huanglongbing in 1982), multiple invasions of ACP, the recent rapid diversification of citrus varieties and extensive commercial cultivation of citrus trees in recent years (Meng *et al.*, 2018).

CLAs strains in Yunnan were highly diversified and the dominant PTG was different from the PTG of LAR. The increased diversity of CLAs could be due to higher prophage activity and increased CLAs genome plasticity, which might in turn be due to its favourable ecological conditions and diversity of citrus hosts. It could also be

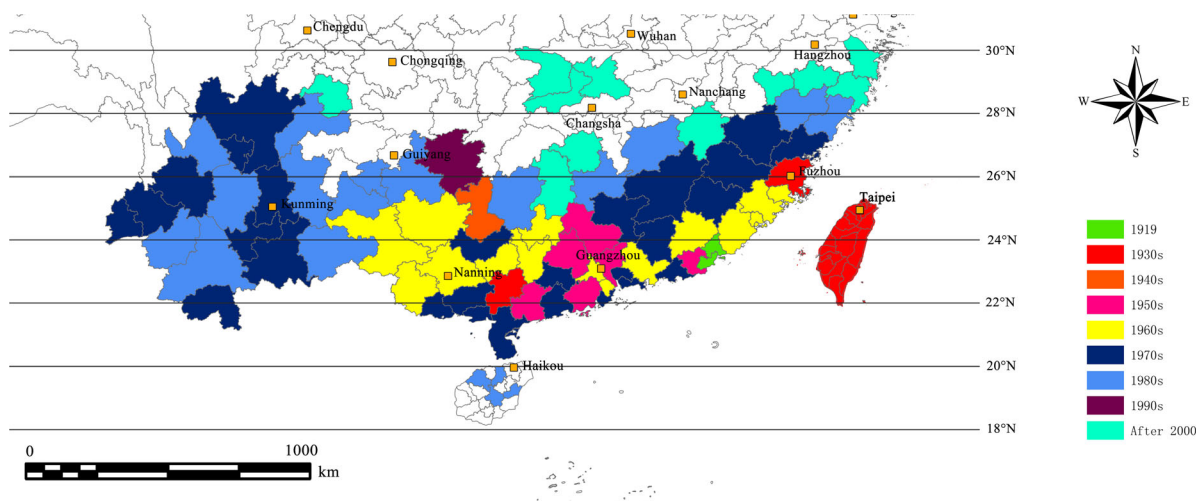


Figure 4 Details of geographic and temporal distribution of huanglongbing (HLB) in China. The maps are divided into counties in each province and are coloured according to when HLB was reported to be prevalent.

attributed to seedling introductions from multiple provinces, including Sichuan, Guangdong, Zhejiang and Hunan in the 1960s to 1970s (Yunnan Citrus Huanglongbing Research Group, 1981, Investigation report on citrus yellowing in Yunnan province, unpublished). Yunnan, as one region of origin of contemporary citrus species (Wu *et al.*, 2018c), is more likely to be a region of origin for HLB in HAR. Potentially, the incursion of HLB in Yunnan was through introductions of seedlings from border countries, such as Laos, Myanmar, Vietnam or Thailand (Bové, 2006), which are geographically close to Xishuangbanna. However, this remains to be confirmed as there is a lack of both historical documents and molecular evidence. The early detections of HLB and psyllids in Sichuan were in Xichang, Ningnan, Dechang and Panzihua (Zhao, 1977), which are adjacent to and share similar ecological conditions with Yunnan. Seeds were commonly used for propagation in Sichuan and seedlings were introduced only from Yunnan with strict quarantine (Zhao, 1977), which is strongly supported by the conserved occurrence of Type 1 prophage and historical documentation. It should be noted that in the LAR, the early occurrence of HLB of subgroup PTG2-1 is associated with lower prophage gene diversity, whereas the late occurrence of PTG2-2 corresponds with higher prophage gene diversity. Thus, as the genetic diversity of HLB was low in Sichuan, it cannot be excluded as a site of origin for HLB in HAR meaning the event might have occurred much earlier than its first report in Yunnan (Zhao, 1977). The diverse prophage profile in Guizhou is related to its unique geographical location, in the middle of four diseased areas, Sichuan, Yunnan, Guangxi and Hunan. The earliest detections of HLB and psyllids in Guizhou province were in Wangmo, Xingyi, Luodian and Ceheng in the early 1980s (Z. T. Chen, unpublished outline of investigation and report of citrus huanglongbing in Guizhou in 1983).

These four locations are adjacent to Guangxi, where psyllids spread rapidly during this period and were probably introduced from Guangxi into southeast Guizhou (Wang, 2002; Guizhou Plant Quarantine Station, 1984, unpublished). Alternatively, the CLas population could also have been restructured through introduction of seedlings from Fujian and Hunan in the 1990s (Professor Jin-qiang Li, Guizhou Provincial Academy of Agricultural Sciences, China, personal communication).

The small proportion of CLas strains without any of the three known prophages could harbour other unknown types of prophage or no prophage at all. It has been demonstrated that the genetic structure and acquisition of CLas in ACP is mediated by the citrus host (Meng *et al.*, 2018; Wu *et al.*, 2018a). Thus, the observation of preferential prophage patterns and their frequency in different provinces could be influenced by the different citrus varieties sampled from different regions in this study, indicating citrus varietal effects cannot be fully ruled out.

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## Data Availability Statement

Data sharing is not applicable to this article as no new data were created or analysed in this study.

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## Supporting Information

Additional Supporting Information may be found in the online version of this article at the publisher's web-site.

**Figure S1.** Geographic and temporal dynamics of huanglongbing (HLB) in low altitude regions in mainland China. The maps are divided into counties or cities in each province and are coloured according to when HLB was reported to be prevalent. White areas indicate no reports of HLB based on documents available.

**Figure S2.** Geographic and temporal dynamics of huanglongbing (HLB) in high altitude regions in mainland China. The maps are divided into counties or cities in each province and are coloured according to when HLB was reported to be prevalent. White areas indicate no reports of HLB based on documents available.

**Figure S3.** Geographic and temporal dynamics of reports of citrus psyllids in six provinces of China. The maps are divided into counties or cities in each province and are coloured according to when psyllids were reported to be prevalent. White areas indicate no reports of citrus psyllids based on data available.

**Figure S4.** Detection of prophage associated genes in 'Candidatus *Liberibacter asiaticus*' (CLAs) strains. (a) Detection of CLAs with primers OI1/OIc2; (b) detection of prophage with primer sets T1-2F/R and T1-3F/R for prophage Type 1, T2-2F/R and T2-3F/R for prophage Type 2, and 891-1F/R and 891-2F/R for prophage Type 3.

**Table S1.** Primers used for intraspecies differentiation.

**Table S2.** Nei's genetic identity (above diagonal) and genetic distance (below diagonal) of strains of 'Candidatus *Liberibacter asiaticus*' from nine provinces in mainland China.