Research Article

Huanglongbing in Bangladesh: A Pilot Study for Disease Incidence, Pathogen Detection, and its Genetic Diversity

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Abstract

Huanglongbing (HLB), also known as citrus greening, is the most serious disease affecting citrus production in Asia, Africa, the Americas, and the Arabian Peninsula. HLB is associated with the α-Proteobacteria "*Candidatus*Liberibacter asiaticus" (*C*Las), "*Ca.* L. africanus" (*C*Laf), and "*Ca.* L. americanus" (*C*Lam). The Bangladesh citrus industry comprises mandarins, sweet oranges, pummelos, limes, and lemons. In 2017-2018, a survey was conducted for two consecutive years in 18 sweet orange growing areas of Bangladesh, and the presence of *C*Las in these areas was confirmed by polymerase chain reaction. HLB incidence and severity were assessed based on leaf symptoms. The results unveiled a widespread prevalence of HLB with incidence ranging between 0.08 and 56% and severity between 1.80 and 28.33. Information on the genetic diversity of *C*Las Bangladeshi isolates was obtained based on the presence or absence of Type 1 (SC1, NC_019549.1) and Type 2 (SC2, NC_019550.1) prophages. *In silico* phylogenetic analyses based on Type 1 and Type 2 prophage sequences showed the presence of four and three clusters of *C*Las isolates, respectively. Combined phylogenetic analyses of Type 1 and Type 2 prophages indicated the existence of four clusters of *C*Las isolates. Bangladeshi *C*Las isolates were found to harbor multiple copies of prophages. The diversity analysis revealed different *C*Las isolates distributed to different citrus growing areas, indicating spread through propagated materials.

Keywords: *Candidatus* Liberibacter asiaticus, Citrus, Survey, Severity

Introduction

The Bangladesh citrus industry is worth more than $100 million and mainly consists of mandarins (*Citrus reticulata*), sweet oranges (*Citrus sinensis*), pummelos (*Citrus maxima*), lemons (*Citrus limon*), and limes (*Citrus aurantifolia*). In Bangladesh, sweet oranges, lemons, and limes have been cultivated on an expanding area since the ancient times around 2500 years ago (Fuller et al. 2017). At present, the main citrus growing areas of Bangladesh are Chattagram, Khagrachari, Rangamati, Bandarban, Noakhali, Sylhet, Moulvibazar, Habigonj, Dhaka, Gazipur, Mymensingh, Kushtia, Dinajpur, Rajshahi, and Rangpur, with a total acreage of approximately 38,060 ha and total production around 165,327 tonnes. Recently, Bangladesh ranked 52 out 133 citrus-producing countries in the world (Knoema 2020). However, Bangladesh does not play a significant role in the citrus world trade. Most of the nation's citrus production is consumed within the country, and only small quantities of limes and lemons are exported to the European Union and Middle East countries. In addition, the recently growing internal demand for mandarins and sweet oranges has been met with imports. To meet Bangladesh's national citrus demand, the Ministry of Agriculture launched a five-year Citrus Development Project (2013-2018). This was then extended for five additional years to increase citrus production by 10-15% on 40,000 ha of land across 27 districts (Personal communication with Dr. Farooque, Project Director, Citrus Development Project, DAE, Government of Bangladesh).

Huanglongbing (HLB), known in East Asia for over a century (Bové 2006), is an emerging disease of citrus in Bangladesh. In Bangladesh, HLB represents a particular threat due to the projected increased cultivation of mandarins and sweet oranges in the country, two citrus types that are especially susceptible to the disease (Islam and Eagle 2016, Tipu et al. 2017; Tipu et al. 2020). HLB symptoms include blotchy chlorosis or mottling of leaves; yellowish shoots; vein corking; stunted growth; poor root growth; small, green, and malformed fruits; and eventually tree death (Bové 2006). The disease is associated with the phloem-limited bacterium '*CandidatusLiberibacter* spp.' (Bové 2006) and is

transmitted by grafting and by the Asian citrus psyllids of the *Diaphorinacitri* insect vector (Bové 2006). Three species, *C*Las, *C*Laf and *C*Lam are known to be pathogenic, with *C*Las being the most widespread (e.g., Asia, Brazil, and North America) (Bové 2006; Teixeira et al. 2005). The first report indicating the possible presence of HLB in Bangladesh dates to 1978 (Catling et al. 1978). The widespread prevalence of the vector *D. citri*, has been reported to correlate with both typical HLB symptoms in citrus trees (i.e. leaf blotchy mottle) in different locations, and with the presence of typical prokaryote-like structures in the phloem sieve tubes of affected plant tissues as detected by enzyme-linked immunosorbent assay (ELISA) (Catling et al. 1978). These results have now been interpreted as an indication of the presence of HLB in Bangladesh in the 1974-76 period (Catling et al. 1978). However, caution should be exercised when interpreting symptoms reported in the past as the symptoms of nutritional deficiency have often been confused with HLB in Bangladesh in the last three to four decades. More recently, HLB has emerged in Bangladesh as the main threat for the expanding cultivation of sweet oranges and mandarins since the presence of HLB associated pathogen, *C*Las, was confirmed by polymerase chain reaction (PCR) in 2017 (Tipu et al. 2017). Since there is still no known solution to HLB, disease management practices, including the use of disease-free nursery stock, early detection of both asymptomatic and symptomatic trees and subsequent removal, and control of the Asian citrus psyllid vector by insecticides, must be implemented to mitigate the threat of this very serious disease (Belasque et al. 2008; Grafton-Cardwell et al. 2013; Hall et al. 2013).

Several molecular markers used to analyze the population diversity of *C*Las have been reported worldwide (Hao et al. 2013; Puttamuk et al. 2014; Pitino et al. 2016). Prophages, the lysogenic form of a bacterial phage where the phage DNA is inserted into the bacterial chromosome, are important genetic elements of the bacterial genome, and play critical roles in bacterial evolution, bacterial cell defense, and environmental adaptation including pathogenesis (Boyd and Brüssow 2002; Feiner et al. 2015). In *C*Las, two types of prophages, Type 1, represented by prophage SC1, and Type 2, represented by prophage SC2, have been described (Zhang et al. 2011; Zheng et al. 2016). SC1 was shown to be involved in the lytic cycle of forming phage particles, and SC2 is thought to be involved in the lysogenic conversion of *C*Las pathogenesis (Fleites et al. 2014; Jain et al. 2015). Among the currently published whole genome sequences of *C*Las from different world geographical regions, eight *C*Las genomes contain extensive prophage sequences, and one *C*Las strain (Ishi71, AP014595.1) from Japan was reported to lack prophage sequences (Duan et al. 2009; Lin et al. 2013; Zheng et al. 2014a, b, 2015; Katoh et al. 2014; Wu et al. 2015a, b; Kunta et al. 2017). Based on PCR experiments with specific primer sets to detect prophage Type 1 and Type 2, *C*Las strains lacking either prophage were also detected in southern China. The current lack of comprehensive studies describing the full repertoire of *C*Las prophages makes it unclear whether these *C*Las strains simply lacked prophages, or they possibly harbored unknown prophages. The presence of more than one prophage in *C*Las raised a question of possible prophage/phage interactions. *C*Las isolates collected in southern China revealed the predominance of a single prophage (Type 1 or Type 2) in any given analyzed *C*Las isolate in the region (Zheng et al. 2016). Type 3 prophage was identified from one sample collected in the Jianxi Province of China (Zheng et al. 2018). Recently, the widespread occurrence of Type 1 and other types of prophages was reported in Brazilian *C*Las strains (Silva et al. 2019). In our study, the genetic diversity of Bangladeshi *C*Las isolates from 14 selected sweet orange growing areas was studied based on the presence and type of prophages detected. We hypothesized that the diverse environmental conditions found in the different citrus producing areas of Bangladesh could have favored pathogen diversity in the country. Information on the genetic diversity of *C*Las isolates from the different geographical locations of the country is required to predict the disease risk. Therefore, an assessment of the status of HLB and information pertaining to the *C*Las population was initiated as a first approach to determine appropriate future management strategies aiming to support the government plan of expanding citrus cultivation in the country.

Materials and Methods

Collection of sweet orange leaves with characteristic HLB symptoms

Suspected HLB symptomatic leaves (i.e. with characteristic blotchy mottle symptoms) were collected from two sweet orange trees in citrus orchards from 18 different Bangladesh locations for a total of 36 samples (6-10 leaves per sample) (**Figure 1**). The sampled orchards were between a minimum altitude of 2 meters above sea level (m. a. s. l.) and a maximum of 1,063 m. a. s. l. Leaf samples were kept in zip-lock bags with silica gel and transported to the Plant Bacteriology and Biotechnology Laboratory, Department of Plant Pathology, Bangladesh Agricultural University, Mymensingh for *C*Las detection by PCR.

Rating of leaf symptoms and calculation of disease incidence and severity

A varying number of sweet orange trees at each of the 18 locations were surveyed visually for leaf symptoms of HLB in September 2017 and 2018 (**Table 1**) as described in Stover and McCollum 2011. Three orchards were surveyed in each location, and the leaf symptom severity (i.e. blotchy mottle) was rated using a 3-point scale (0, 1, 2) with 0 representing no apparent HLB symptoms, 1 suspect HLB symptoms, and 2 likely HLB symptoms (Stover and McCollum, 2011).

The incidence of HLB was calculated using the following formula:

$$\% \text{ HLB symptoms bearing plant} = \frac{\text{Number of plants with suspected and likely HLB leaf symptoms in each orchard}}{\text{Total number of plants in each orchard}} \times 100$$

HLB severity was calculated using the formula after (Ahmad et al. (2011):

$$\% \text{ HLB severity} = \frac{X1 + X2 + \cdots + Xn}{Y \times \text{maximum rating scale}} \times 100$$

Where, $X_{1...n}$ was scored of HLB leaf severity symptoms of each plant and Y = total number of plants.

Leaf tissue processing and genomic DNA extraction

The 6-10 leaves collected from the two trees in each of the 18 locations were combined into a single leaf sample for DNA extraction and downstream *C*Las detection by PCR (18 samples, one per location, **Figure 3**).

Leaves were washed with sterile distilled water and 70% ethanol and dried on blotting paper to remove excess water. Leaf midribs were removed and chopped with sanitized scissors. Approximately 60 mg of leaf tissue were flash frozen in liquid nitrogen in a micro centrifuge tube, and ground into a fine powder using a micro pestle. The genomic DNA was extracted using the Wizard Genomic DNA purification kit (Promega, Madison, WI, USA) in accordance with the manufacturer's instructions.

*Detection of C*Las *by PCR*

*C*Las was detected by PCR using primers Las606 (5´-GGAGAGGTGAGTGGAATTCCGA-3´), and LSS (5´-ACCCAACATCTAGGTAAAAACC-3´) as described previously by Fujikawa and Iwanami (2012). The PCR reactions were performed using GoTaq® Green Master Mix (Promega, Madison, USA) in 25 µl reaction mixtures containing 12.5 µlGoTaq® Green Master Mix 12.5 µl of GoTaq® Green Master Mix (1X Green GoTaq® Reaction Buffer (pH 8.5), 200 µM of dNTPs each, 1.5 mM MgCl2 and two dyes blue and yellow), 1 µl of 1 µM of each forward and reverse primers, 1 µl (100 ng/µl) template DNA and 9.5µl of ddH_2O. The PCR conditions were 9 min of pre denaturation at 96° C, followed by 35 cycles of 30 s denaturation at 96° C, 30 s annealing at 55° C, 1 min extension at 72° C, and then a single final extension step of 7 min at 72° C. The amplified *C*Las DNA fragments (expected size 500 bp) were visualized after electrophoresis on 1.2 % agarose gel.

*Analysis of C*Las *genetic diversity using Type 1 and Type 2 prophages.*

Fourteen (14) *C*Las isolates, selected based on the geographical similarity of the locations they were isolated from in Bangladesh, were analyzed for *C*Las genetic diversity. Genetic diversity of these 14 *C*Las was analyzed using previously published primers for prophages (Zheng et al. 2016) (**Table 1**). Standard PCRs were performed on a Bio-Rad T100™ Thermal Cycler (Bio-Rad, Hercules, CA, USA) in 25 µl reaction volume containing 12.5 µl of GoTaq® Green Master Mix (1X Green GoTaq® Reaction Buffer (pH 8.5), 200 µM of dNTPs each, 1.5 mM MgCl2 and two dyes blue and yellow), 1 µl of 10 µM of each of the forward and reverse primer, template DNA 1 µl (100 ng/µl) and 9.5 µl of ddH_2O. PCR was performed with initial denaturation at 96° C for 5 min, 35 cycles of amplification (94° C for 30 s, 60° C for 30 s, and 72° C for 60 s) and ended with a final extension of 72° C for 10 min.

Table 1. General information of primers specific to *C*Las Type 1 and Type 2 prophages (Zheng et al. 2016)

Primer Set (F/R)	Sequence (5´-3') set F/R	Amplicon size (bp)	Locus name	Putative function	Prophage Type
SC1-2F/SC1-2R	TGGCTCGGGTTCAGGTAAAT AAGGGCGACGCATGTATTTC	975	SC1_gp035	Endolysin	1
SC1-3F/SC1-3R	CTCACTGCGTCTTGATTCGG CGAACGAGCGGTATGTTTGT	866	SC1_gp050	Phage-related protein	1
SC2-2F/SC2-2R	ACCCTCGCACCATCATGTTA TCGTCTTGATTGGGCAGAGT	813	SC2_gp030	Structural protein	2
SC2-3F/SC2-3R	ACAGTTAAGAGCCACGGTGA AAGACGTGGGTGTTATGGGT	918	SC2_gp040	Phage-related protein	2

Visualization of PCR products

The PCR products were run and visualized on 1% TBE agarose gel containing 0.2µg ethidium bromide per 100 ml gel from stock solution. Following electrophoresis, the gel was placed under a UV transilluminator (GelView Master, Dynamica, UK) to visualize the amplified DNA bands.

Phylogenetic analyses

The size of all distinct bands observed on gels was determined according to their position on the gel relative to the DNA marker (Tsingke, Biological Technology, China). The expected fragment size scored visually. The scores obtained using all primers in the analysis were then pooled to create a single data matrix. This was used to construct an Unweighted Pair Group Method of Arithmetic Means (a UPGMA) dendrogram among populations using the NTSYS-pc Version. 2.02i Numerical Taxonomy and Multivariate Analysis System (Applied Biostatistics Inc., Exeter Software, Setauket, New York) (Rolf 1997).

Results

Assessment of HLB incidence and severity in sweet orange trees in Bangladesh

HLB incidence and severity in Bangladesh were assessed visually based on leaf symptom severity (i.e. blotchy mottle) in sweet orange field trees surveyed in 18 citrus growing areas (**Figure 1**). HLB incidence and severity ratings for individual trees at each location (Table 2) were recorded and used to calculate incidence and severity at each geographical location assessed in this study (**Figure 2**). The highest percentage of HLB incidence was found in Rangamati (56.67%) and Jamalpur (56.67%), followed by Bandarban (40%), and

the lowest incidence was observed in Panchagarh (3.61%), while slightly higher incidence values were recorded in other areas: Tangail (4.16%), Cumilla (4.16%), Mymensingh (4.16%), Gaibandha (5.41%), Thakurgaon (5.41%), Habiganj (5.83%), Gazipur (6.11%), Chattagram (7.5%), Khagrachari (8.11%), Sylhet (10%), Netrokona (10.83%), Moulavibazar (10.83%), Sherpur (10.83%) and Narsingdi (10.83%) (**Figure 2A**). HLB severity in different locations ranged from 1.80 to 28.33%. The maximum HLB severity was observed in Jamalpur and Rangamati (28.33%) followed by Bandarban (20%), and the lowest (1.80%) severity was recorded in Panchagarh followed by Tangail, Cumilla, Mymensingh, Gaibandha, Thakurgaon (all at 2.70%), Habiganj (2.91%), Gazipur (3.05%), Chattogram (3.75%), Khagrachari (4.0%), Sylhet (5%), Netrokona, Moulavibazar, Sherpur and Narsingdi (all at 5.41%) (**Figure 2B**).

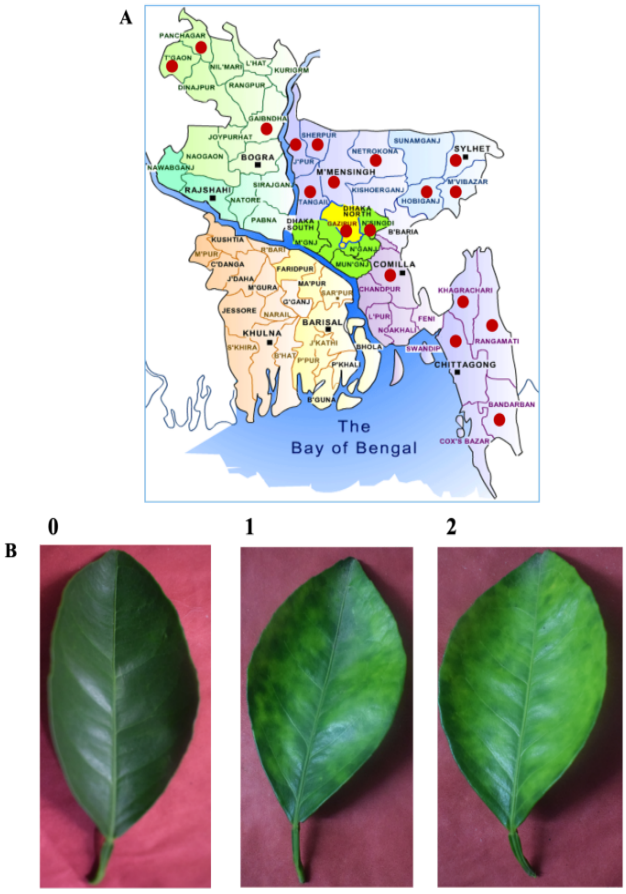

Figure 1. A) Map showing the surveyed geographical locations to assess the status of HLB. Red circles indicate the locations where the surveys were conducted. **B)** HLB leaf severity scale: 0, No apparent HLB symptoms (left); 1, Suspected HLB symptoms (middle) and 2, Likely HLB symptoms (right).

Table 2. Number of HLB asymptomatic and symptomatic trees based on the severity rating at the 18 different surveyed locations.

Location	*Number of trees based on HLB severity rating (0,1, 2)			Total number of trees examined
	0	1	2	
Panchagarh	328	11	1	340
Thakurgaon	265	7	8	280
Narsinghdi	250	20	10	280
Gaibhanda	265	5	10	280
Sherpur	250	10	20	280
Jamalpur	110	136	4	250
Mymensingh	565	7	8	580
Gazipur	285	9	6	300
Cumilla	385	4	11	400
Tangail	565	14	1	580
Moulavibazar	250	2	28	280
Sylhet	270	10	20	300
Khagrachari	280	10	10	300
Rangamati	110	4	136	250
Bandarban	180	12	108	300
Chattogram	370	10	20	400
Habiganj	375	7	18	400
Netrokona	250	20	10	280

*A 3-point severity scale (0, 1, 2): 0 = no apparent HLB symptoms, 1 = suspect HLB symptoms, and 2=likely HLB symptoms (Stover and McCollum, 2011).

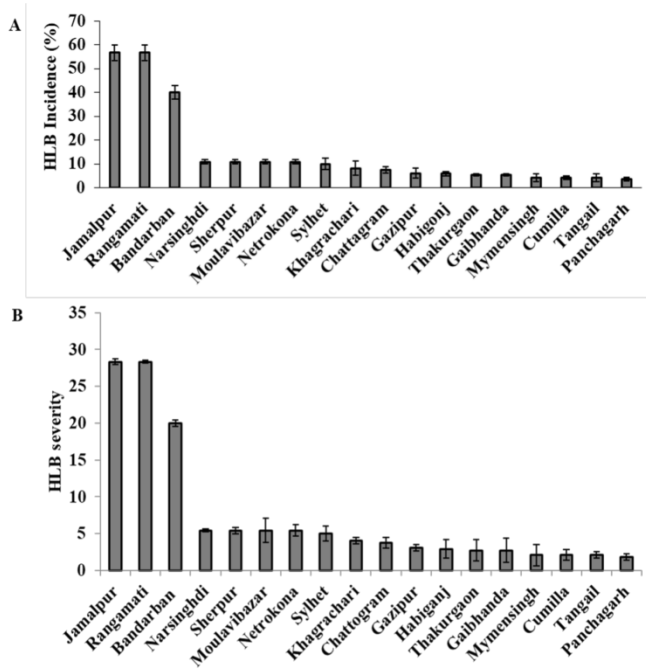

Figure 2. HLB incidence and severity in the different surveyed locations. **A)** Percentage of symptomatic plants (incidence) and **B)** HLB severity. Trees displaying severity =1 or 2 on the 3-point scale, where 0= no apparent HLB symptoms, 1= suspect HLB symptom, and 2= likely HLB symptoms, were included in the calculation. Three orchards were surveyed at each location. Error bars represent standard error.

*PCR-based detection of C*Las

HLB leaf symptoms observed visually in the sweet orange field trees were correlated with the HLB associated bacterium *C*Las by PCR. PCR, using the primer pairs Las606/LSS, was performed on the DNA extracted from symptomatic sweet orange leaves collected from the 18 different locations surveyed in this study. The presence of *C*Las was detected in 15 out of the 18 assessed locations: Panchagarh, Thakurgaon, Gaibandha,

Sherpur, Jamalpur, Mymensingh, Gazipur, Cumilla, Tangail, Moulavibazar, Sylhet, Khagrachari, Rangamati, Bandarban, Chattagram. However, no *C*Las-amplification was obtained from the HLB symptomatic leaves collected from Narsinghdi, Habiganj and Netrokona (**Figure 3**).

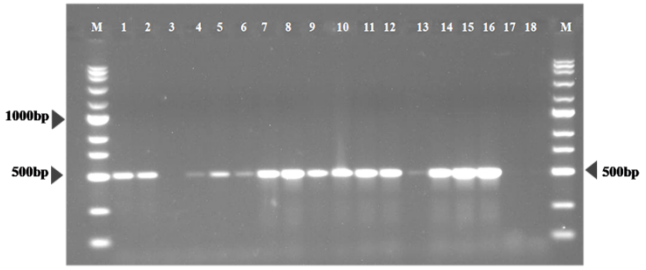

Figure 3. Agarose gel electrophoresis of HLB symptomatic leaf DNA amplified with *Candidatus*Liberibacter spp. primer pair Las606 and LSS. Lane M: 250bp DNA ladder; lane 1: Panchagarh, lane 2: Thakurgaon, lane 3: Narshingdi, lane 4: Gaibandha, lane 5: Sherpur, lanes 6: Jamalpur, lanes 7: Mymensingh, Lane 8: Gazipur, Lane 9: Cumilla, Lane 10: Tangail, lane 11: Moulavibazar, lane 12: Sylhet, lane 13: Khagrachari, lane 14: Rangamati, lane 15: Bandarban, lane 16: Chattogram, lane 17: Habiganj and lane 18: Netrokona.

*Assessment of C*Las *genetic diversity using prophages*

After the initial survey for the presence of *C*Las in 18 citrus growing locations of Bangladesh (**Figure 3**), *C*Las isolates were selected from 14 locations representing the northern part (Panchagarh and Thakurgaon), mid-eastern part (Mymensingh 01 (Haluaghat), Mymensingh 02 (Bhaluka) and Mymensingh 03 (BAU Farm), Gazipur and Cumilla), medium hilly eastern part (Sylhet, Habiganj and Moulvibazar), and the hilly citrus growing areas (Chattagram, Khagrachari, Rangamati and Bandarban) of Bangladesh for analyses of genetic diversity using prophages Type 1 and Type 2. Detection of *C*Las from the symptomatic leaves collected from these 14 citrus growing areas was confirmed by PCR (**Figure 4**).

Of the 14 analyzed *C*Las isolates, 8 (57%) were positive for the presence of Type 1 prophage: 6 isolates generated a 975bp band with primer set SC1-2F/SC1-2R (**Figure 5A**), and 3 isolates generated a 866bp band with primer set SC1-3F/SC1-3R (**Figure 5B**), with 2 isolates (lanes 2 and 6) showing a band with both primer sets. Phylogenetic analyses of *C*Las isolates based on the presence or absence of prophage Type 1 showed the analyzed *C*Las strains distributed to two main clusters. Cluster I consists of two sub-clusters. Sub-cluster I included *C*Las isolates obtained from Panchagarh, Mymensingh 02 (Bhaluka), Sylhet, Cumilla, Chattagram and Gazipur, and sub-cluster II consists of *C*Las isolates obtained from Thakurgaon, Bandarban and Rangamati. Cluster II includes two sub-clusters; sub-cluster III represents *C*Las strains obtained from Mymensingh 01 (Haluaghat), Mymensingh 03 (BAU farm), Habiganj, and sub-cluster IV consists of *C*Las isolates obtained from Moulvibazar and Khagrachhari (**Figure 5C**).

PCR amplification showed the presence of prophage Type 2 (813 bp fragment with primers SC2-2F/SC2-2R, and a 918 bp fragment with primers SC2-3F/SC2-3R) in 14% (3 out of 14) of the 14 analyzed citrus growing areas (**Figure 6A** and **6B**). Phylogenetic analysis based on the presence or absence of prophage Type 2 revealed two clusters of *C*Las strains. Cluster I consists of two sub-clusters. Sub-cluster I includes *C*Las isolates obtained from Panchagarh, Thakurgaon, Mymensingh 01 (Haluaghat), Mymensingh 02 (Bhaluka), Mymensingh 03 (BAU Farm), Habiganj, Moulvibazar, Cumilla, Chattagram, Rangamati and Gazipur and sub-cluster II consists of *C*Las isolates from Bandarban. Cluster II consists of *C*Las isolates from Sylhet and Khagrachari (**Figure 6C**).

The genetic relationship among the different *C*Las isolates included in this study based on the presence or absence of prophage Type 1 and Type 2 revealed *C*Las isolates from 14 citrus growing areas distributed to four clusters (**Figure 7A**). Interestingly, some *C*Las isolates obtained from Khagrachari and Bandarban were found to possess both prophage Type 1 and Type 2. Prophage Type 1 and Type 2 were found to geographically coexist in some growing areas, indicating at least two independent introductions of *C*Las in some growing areas of Bangladesh (**Figure 7B** and **7C**).

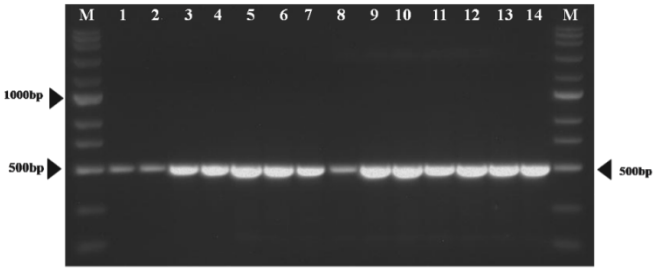

Figure 4. PCR detection of *C*Las isolates for genetic diversity analyses from HLB symptomatic leaves collected from 14 selected citrus growing areas used. Lane M: 250bp DNA ladder; 1.Panchagarh, 2.Thakurgaon, 3.Mymensingh 01 (Haluaghat), 4. Mymensingh 02 (Bhaluka), 5.Mymensingh 03 (BAU Farm), 6. Habiganj, 7.Moulvibazar, 8.Sylhet, 9.Cumilla, 10. Chattogram, 11.Rangamati, 12.Khagrachhari, 13.Bandarban and 14.Gazipur.

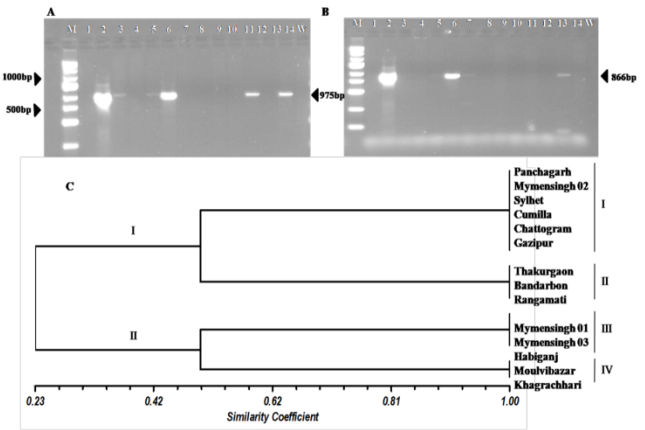

Figure 5. PCR amplification of prophage Type 1 using primers SC1-2F/SC1-2R **(A)** and SC1-3F/SC1-3R **(B).** M: 250bp DNA ladder; W: Water; 1-14: *C*Las strains obtained from selected sweet orange growing areas of Bangladesh: 1.Panchagarh, 2.Thakurgaon, 3.Mymensingh 01 (Haluaghat), 4.Mymensingh 02 (Bhaluka), 5.Mymensingh 03 (BAU Farm), 6.Habiganj, 7.Moulvibazar, 8.Sylhet, 9.Cumilla, 10.Chattogram, 11.Rangamati, 12.Khagrachhari, 13.Bandarban and 14.Gazipur. **C)** Phylogenetic tree showing the genetic relationship of *C*Las isolates obtained from 14 selected sweet orange growing areas of Bangladesh

(from Panchagarh, Thakurgaon, Mymensingh 01 (Haluaghat), Mymensingh 02 (Bhaluka), Mymensingh 03 (BAU Farm), Habiganj, Moulvibazar, Sylhet, Cumilla, Chattogram, Rangamati, Khagrachhari Bandarban and Gazipur based on Type 1 prophages.

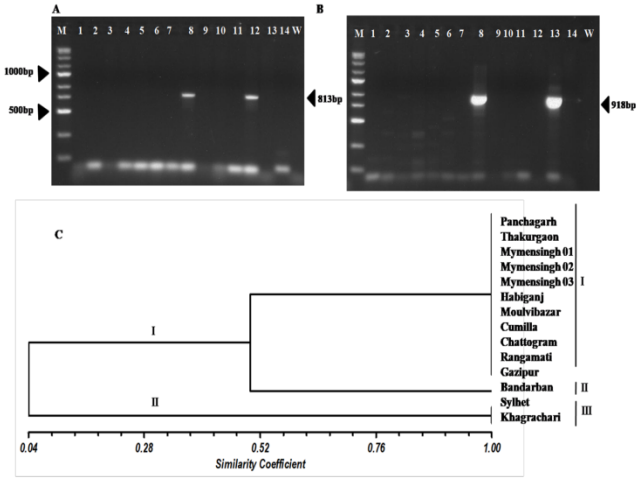

Figure 6. PCR amplification of prophage type 2 using primers SC2-2F/SC2-2R **(A)** and SC2-3F/SC2-3R **(B).** M: 250bp DNA ladder, W: Water, 1-14: *C*Las strains obtained from 14 selected sweet orange growing areas of Bangladesh. 1.Panchagarh, 2.Thakurgaon, 3.Mymensingh 01 (Haluaghat), 4.Mymensingh 02 (Valuka), 5.Mymensingh 03 (BAU), 6.Habiganj, 7.Moulvibazar, 8.Sylhet, 9.Cumilla, 10.Chattagram, 11.Rangamati, 12.Khagrachhari, 13.Bandarban and 14.Gazipur and C) Phylogenetic trees showing the genetic relationship of *C*Las isolates obtained from selected sweet orange growing areas of Bangladesh (Panchagarh, Thakurgaon, Mymensingh 01 (Haluaghat), Mymensingh 02 (Bhaluka), Mymensingh 03 (BAU Farm), Habiganj, Moulvibazar, Sylhet, Cumilla, Chattogram, Rangamati, Khagrachhari, Bandarban and Gazipur based on Type 2 prophages.

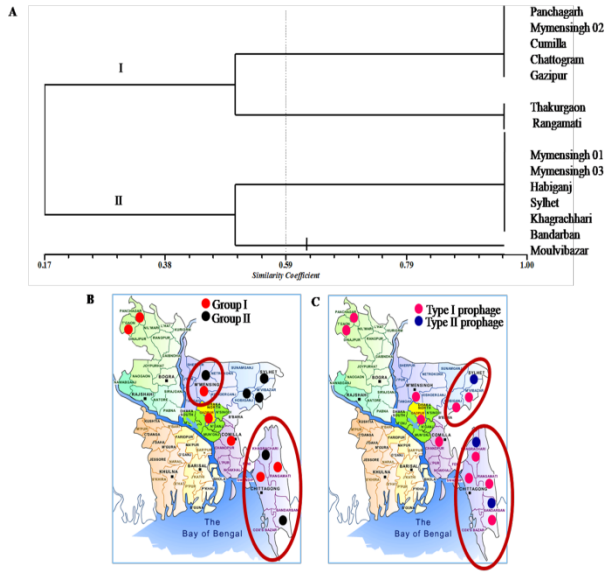

Figure 7. A) Phylogenetic relationship of *C*Las isolates obtained from 14 selected sweet orange growing areas of Bangladesh based on the presence or absence of prophage Type 1 and Type 2. Groups I and II indicate phylogenetic groups categorized based on the presence and absence of Type I and Type 2 prophages. *C*Las isolates used from Panchagarh, Thakurgaon, Mymensingh 01 (Haluaghat), Mymensingh 02 (Bhaluka), Mymensingh 03 (BAU Farm), Habiganj, Moulvibazar, Sylhet, Cumilla, Chattogram, Rangamati, Khagrachari, Bandarban and Gazipur. **B)** Geographical distribution of phylogenetic groups (I & II) of *C*Las isolates possessing prophage Type 1 and Type 2 prophages in the same region. **C)** Geographical distribution of *C*Las isolates carrying both prophage Type 1 and Type 2. Circles indicate the coexistence of two prophages (Type 1 and Type 2) in the same region.

Discussion

HLB has emerged as an increasing threat to the expansion of citrus cultivation in Bangladesh. A field survey was conducted to assess visually the status of HLB (incidence and severity) in sweet orange trees in 18 citrus growing areas of Bangladesh. HLB incidence and severity were assessed based on visual observations in the field, and symptomatic leaf samples were collected from the locations of interest for PCR-based detection of *C*Las. The observed HLB leaf symptoms included blotchy mottling on leaves in agreement with the most characteristic HLB symptoms associated with *C*Las described previously (Lin 1956; Schneider 1968; da Graça 1991; Jagoueix et al. 1994; Bové 2006).

The suspected HLB symptomatic field sweet orange leaves were tested by PCR using 16S rDNA-based primers as previously reported (Fujikawa and Iwanami 2012; Fujikawa et al. 2013) and *C*Las infection was confirmed in 15 of the 18 surveyed locations. Interestingly, symptomatic leaf samples from three different locations (Narshingdi Habiganj, Netrokona) tested negative for *C*Las This could have resulted from several reasons including low bacterial titers, uneven pathogen distribution, and the use of conventional PCR (Figure 3). Our results indicate the challenges related to HLB diagnosis in the field. A combination of visual observations and laboratory analysis with the most sensitive molecular pathogen detection tools such as quantitative PCR (qPCR) or digital qPCR offers a very effective approach for HLB diagnosis (Gottwald et al. 2007; Li et al. 2006; Li et al. 2007; Selvaraj et al. 2018; Zheng et al. 2016).

The HLB status assessment performed in this study indicated that HLB represents an increasing threat to the Bangladesh citrus industry overall. In particular, the growing mandarin and sweet orange Bangladesh industries are at high risk since these citrus types are highly susceptible to HLB. The sweet orange orchards located in Rangamati (hilly areas adjacent to the Indian border) and Jabalpur displayed the highest incidence of HLB with the altitude of 17m as well as 12m respectively, and the lowest level of incidence of 3.61% showed in Panchagarh (60m). A moderate level of infection (40%) was found in the area ofBandarbhan with an elevation of 1063.142m. However, the area of Narsinghdi (4m), Sherpur (3m), Moulovibajar (13m), Netrokona (2m) remained at the same level with 10.83% of incidence. In addition, 6-10% incidence was found in Gazipur (4m), Chattogram (7m), Khagrachari (48m), and Sylhet (9m). The lowest level of observed incidence ranged from 4.16 to 5.83% in six districts: Mymensingh (17m), Cumilla (12m), Tangail (13m), Habigonj (6m), Thakurgoan (37m), Gaibandha (27m). In terms of severity, the peak was found in the area of Jamalpur (12m) and Rangamati (17m), a moderate level of severity was observed in

Bandarbhan (1063.142m), and the lowest level of severity was found in Panchagarh (60m). In addition, the medium level of HLB severity (5.41%) was manifested in the area of Narsinghdi (4m), Sherpur (3m), Moulovibajar (13m), Netrokona (2m). A severity ranging from 3.05 to 5.0% was seen in the location of Gazipur (4m), Chattogram (7m), Khagrachari (48m), Sylhet (9m). The lower level of severity was found in six districts including Habigonj (6m), Thakurgoan (37m), Gaibandha (27m), Mymensingh (17m), Cumilla (12m), Tangail (13m). A previous survey in Bangladesh found the highest HLB incidence to be 3.33% with a highest severity of 10% (Rahman 2011). HLB is known to occur at lower elevations (360 m) under low humidity and at both cool and warm temperatures up to 35°C (Garnier and Bové 1993). However, no clear relationship was observed between altitude and either HLB incidence or HLB severity in this study. Mild to severe symptoms of HLB were observed in different citrus growing agro-ecologies across a range of altitudes from low (<1000 m. a. s. l) to high (>1500 m. a. s. l) in Ethiopia, Kenya and Uganda (Ajene et al. 2020). HLB symptoms in the Ethiopian highlands appeared to be severe, in contrast to reports that they are less pronounced and disappear above 1,500 m (Aubert, 1987). The higher incidence and severity reported in our study may be due to the genetic variation of *C*Las strains, sources of the planting materials, environmental effects and management practices adopted in that particular agro-ecological zone as well as the presence of the ACP insect vector, in particular in the areas very close to Indian border.

In this study, the genetic diversity of *C*Las isolates obtained from sweet orange orchards of 14 selected citrus growing areas of Bangladesh locations was assessed based on the presence of *C*las Type 1 and Type 2 prophages. Prophages, the lysogenic form of bacterial phages, are important genetic entities of *C*Las. *C*Las prophages may play an important role in HLB disease development, similar to the prophage roles observed with other plant pathogenic bacteria including *Ralstonia solanacearum* (Yamada et al. 2007) and *Xylella fastidiosa* (Summer et al. 2010). Studies on the genetic diversity of *C*Las strains have been performed utilizing prophage gene sequences as discriminants. Examples include *Ca.* L. asiaticus strain without prophage (Katoh et al. 2014), with a single prophage (Zheng et al. 2014), two prophages (Duan et al. 2009; Lin et al. 2013; Zhang et al. 2011), and even three prophages (Zheng et al. 2018). In this study, analysis of *C*Las genetic diversity with primers related to prophage Type 1 showed presence of Type 1 in 50% of the analyzed isolates (7 out of 14), and with four clusters distributed to different citrus growing areas (Figure 4C). In contrast, only 14% of analyzed *C*Las isolates contained Type 2 prophage, with three clusters of *C*Las isolates (Fig 5C). Furthermore, analysis of the phylogenetic relationship of *C*Las isolates revealed four clusters of *C*Las isolates based on the presence and absence of both Type 1 and Type 2 prophages. These results showed that *C*Las strains containing either both Type 1 and Type 2 prophages or either Type 1 or Type 2 prophages distributed to different locations of the country (Figure 6B-C). Phage was previously reported in association with HLB disease with *Ca.*Liberibacter species (Fu et al. 2015), and the presence of a circular replicating form of both SC1 and SC2 in *C*Las strains was also reported (Zhang et al. 2011). In addition, the lack of detection of Type 1 or Type 2 prophages in *C*Las was previously interpreted as indicating the absence of a prophage (Katohet al. 2014; Zheng et al. 2016). More recently, *C*Las strains isolated from *Diaphorinacitri* from seven localities in the Jiangxi province, China, contained only one prophage (67%), and *C*Las strains with two and no prophage types accounted for 12% and 21% (Lifen et al. 2017). Zheng et al. (2016) reported that the genome sequence analysis of *C*Las Strain A4 from Guangdong contained only a Type 2 prophage which was predominant in the bacterial population (82.6%). Out of 523 *C*Las DNA samples extracted from the leaf petioles of citrus from southern China, only 18 samples were found to lack both prophages (Zheng et al. 2017). In addition to Type 1 and Type 2, other *C*Las prophages have been reported (Zhou et al. 2014) including a recently described Type 3 prophage (Zheng et al. 2018). Here, our results indicated a high variability among *C*Las Bangladeshi isolates in terms of presence or absence of Type 1 and Type 2 prophages. Absence of prophage Type 1 and Type 2 prophages in most of the *C*Las Bangladeshi isolates obtained from Panchagarh, Mymensingh 02 (Bhaluka), Cumilla, Chattagram and Rangamati indicated either the partial presence of these prophages or sequence variation that precludes PCR amplification and detection (Silva et al. 2019). In addition, presence of additional *C*Las prophages should be tested in the *C*Las isolates from Bangladesh collected from different growing areas in this study.

In summary, this work provides preliminary information regarding the incidence of HLB in Bangladesh in sweet orange trees. Additional surveys will be needed to assess the status of HLB in other hosts of the *Rutaceae* family to include new citrus growing areas. The origin and evolution of *C*Las in Bangladesh is still unknown. However, PCR based detection for the presence or absence of prophages in *C*Las genome indicated genetic variation among *C*Las strains exists in Bangladesh. *C*Las isolates possessing both prophage Type 1 and Type 2 prophages or either Type 1 or Type 2 were found to geographically coexist in some citrus growing areas, indicating at least two independent *C*Las introduction events in those areas (Figure 6B & 6C). In the future, whole genome sequence analysis of Bangladeshi *C*Las strains using next-generation sequencing technologies will shed light on the diversity of prophages and *C*Las population structure in Bangladesh and other Asian countries. The high HLB incidence in some of the sweet orange growing areas found in this study warns citrus researchers and growers of the high risks posed by HLB in the context of an expanding sweet orange and mandarin citrus industry in Bangladesh.

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