Genetic Diversity of the Indian Populations of '*Candidatus Liberibacter asiaticus*' Based on the Tandem Repeat Variability in a Genomic Locus

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Accepted for publication 3 March 2015.

ABSTRACT

Ghosh, D. K., Bose, S., Motghare, M., Warghane, A., Mukherjee, K., Ghosh, D. K., Sr., Sharma, A. K., Ladaniya, M. S., and Gowda, S. 2015. Genetic diversity of the Indian populations of '*Candidatus liberibacter asiaticus*' based on the tandem repeat variability in a genomic locus. *Phytopathology* 105:1043-1049.

Citrus huanglongbing (HLB, citrus greening disease) is an extremely destructive disease affecting citrus and causes severe economic loss to the crop yield worldwide. The disease is caused by a phloem-limited, noncultured, gram-negative bacteria *Candidatus Liberibacter* spp., the widely present and most destructive species being '*Candidatus Liberibacter asiaticus*'. Although the disease has been reported from almost all citrus growing regions of India, knowledge on the molecular variability of the pathogen '*Ca. L. asiaticus*' populations from different geographical regions and cultivars is limited. In the present study, variability of the Indian '*Ca. L.*

asiaticus' based on the tandem repeats at the genomic locus CLIBASIA_01645 was characterized and categorized into four classes based on the tandem repeat number (TRN); Class I (TRN ≤ 5), Class II (TRN > 5 ≤ 10), Class III (TRN > 10 ≤ 15), and Class IV (TRN > 15). The study revealed that the Indian population of '*Ca. L. asiaticus*' is more diverse than reported for Florida and Guangdong populations, which showed less diversity. While Florida and Guangdong populations were dominated by a TRN5 and TRN7 genotype, respectively, the Indian '*Ca. L. asiaticus*' populations with TRN copy numbers 9, 10, 11, 12, and 13 were widely distributed throughout the country. Additionally, TRN2 and TRN17 genotypes were also observed among the Indian '*Ca. L. asiaticus*' populations. The predominant '*Ca. L. asiaticus*' genotypes from the northeastern region of India were TRN6 and TRN7 (53.12%) and surprisingly similar to neighboring South China populations. Preliminary results showed absence of preference of citrus cultivars to any specific '*Ca. L. asiaticus*' genotype.

Citrus, a native to China, has been growing in India since 2500 BC (1,4,27). Among all diseases affecting citrus crop globally, huanglongbing (HLB) is considered the most destructive disease. Estimates have suggested that over 60 million citrus trees have been destroyed worldwide by this disease alone (3,18). The disease was first noted in China in the late 1800s (24) and was confirmed in India in 1967 when a successful transmission of the disease with the Asian citrus psyllid was achieved (4,5,12,36). HLB has also been reported in São Paulo in Brazil and Florida, Texas, and California in the United States (17,18). It is of great concern to the worldwide citrus industry (8,17,32). The disease affects all commercial citrus cultivars including preferred root stocks.

HLB is associated with the *Candidatus Liberibacter* spp., a phloem-limited, gram-negative, as yet not cultured alpha-proteobacterium. Based on geographic location of their origin, temperature tolerance and sequence variations in 16S rRNA region, they are classified into three species: '*Ca. L. asiaticus*', '*Ca. L. africanus*', and '*Ca. L. americanus*' (3,22). The pathogen is transmitted by psyllid vectors, but differs in their vector species specificity and the environment. '*Ca. L. asiaticus*' and '*Ca. L. americanus*' are heat-tolerant and

vectored by *Diaphorina citri* Kuwayama, while '*Ca. L. africanus*' is heat-sensitive and vectored by *Trioza erytreae* Del Guercio (3,5). In the Indian subcontinent, '*Ca. L. asiaticus*' is the most prevalent species.

Methods for distinguishing '*Ca. L. asiaticus*' isolates are important for understanding the genetic structure of pathogen populations and their ecological and epidemiological studies. Initially, conserved genomic loci such as 16S rDNA sequences were used to analyze the populations of '*Ca. L. asiaticus*' (22). Subsequently, the loci of 16S rRNA gene, β -operon in ribosomal protein genes (*rplAJ*), and an outer membrane protein gene (*omp*) in '*Ca. L. asiaticus*' were compared (2,9,10). However, sequence analysis revealed very limited variations in these genes (9,10,30). In general, the extent of genetic variations at these loci (16S rDNA, *rplAJ* gene, and *omp* gene) was low, rendering it difficult to characterize bacterial populations of different origins with adequate resolution. However, recent studies have focused on more variable loci as complete '*Ca. L. asiaticus*' genome sequence become available (12). Tomimura et al. (33) analyzed the single nucleotide polymorphisms (SNPs) in a bacteriophage-type DNA polymerase gene and revealed three clusters of '*Ca. L. asiaticus*' strains from Southeast Asia. Focusing on the '*Ca. L. asiaticus*' populations in China, Liu et al. (25) analyzed the variation of a prophage gene and found that the gene frequencies were significantly different between bacterial populations from Guangdong and Yunnan provinces. A unique genomic region of '*Ca. L. asiaticus*' showing locus mosaicism was recently characterized that revealed that '*Ca. L. asiaticus*' isolates in China

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*The e-Xtra logo stands for "electronic extra" and indicates that one supplementary figure and two supplementary tables are published online.

<http://dx.doi.org/10.1094/PHYTO-09-14-0253-R>
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could be divided into high and low altitude groups (35). Similarly, the availability of complete genome sequence (1.23 Mb) of the pathogenic '*Ca. L. asiaticus*' strain psy62 (12) allowed diversity analysis of the bacterial populations based on tandem repeat numbers (TRN) of few genomic loci. Simple sequence repeat markers, also known as microsatellites, are tandem repetitive DNA sequences with repeat motif lengths of 2 bp or more (34). In general, the variability of the repeats are believed to be caused by slipped-strand mispairing (31), the genetic instability of polynucleotide tracts, especially poly (G-T) (19), and DNA recombination between homologous repeat sequences (34). Simple sequence repeat with a potential variable number of tandem repeats in bacterial DNA have been used as markers for differentiating and subtyping strains of several bacterial species including *Xylella fastidiosa* (7), which causes variegated chlorosis of citrus and Pierce's disease in grapevines (23,26). Islam et al. (20) developed microsatellite markers and identified three major genetic groups of '*Ca. L. asiaticus*' worldwide. Chen et al. (6) studied the variation analysis of TRN at a genomic locus (CLIBASIA_01645) of '*Ca. L. asiaticus*' strains in Guangdong, China and Florida and observed that the Florida bacterial population was dominated by a TRN = 5 genotype (84.5%), while the Guangdong bacterial population predominantly contained a TRN = 7 genotype, albeit at 47.6%. Katoh et al. (23) identified four tandem repeat loci showing high sensitivity in detection of '*Ca. L. asiaticus*' diversity among isolates from Japan, Taiwan, and Indonesia. Locus CLIBASIA_01645 (designated as 005) was one of them with highest overall Nei's diversity value (23). Recently, Deng et al. (11) made double-locus analyses and evaluated genomic variations of '*Ca. L. asiaticus*' populations based on the analyses of two genomic loci: *trnI*, characteristic of variable TRNs and *snp1*, characteristic of SNPs.

HLB, known by different names in different countries, was familiar as citrus dieback in India since the eighteenth century and was attributed to several factors including soil disorders, nutritional deficiencies, involvement of fungi and viruses (4). In view of the serious nature of the citrus dieback problem and its economic importance to Indian citriculture, the Indian Government in the year 1965 invited Lilian Fraser, an Australian scientist to investigate the possible cause of citrus dieback in the country. After visiting the major citrus growing regions of India, she concluded that the dieback affecting citrus trees in India carry the identical symptoms of HLB seen in South Africa (13). However, the experimental proof of the presence of HLB in India came in 1967 when the successful transmission of HLB agent was obtained by the insect vector, citrus psylla (*Diaphorina citri*) (5). Subsequently, several reports confirm the wide-spread distribution of HLB and its insect vector in all citrus growing states of India including Maharashtra, Andhra Pradesh, Punjab, Tamil Nadu, West Bengal (Darjeeling Hills), and Bihar as well as the northeastern states of Assam, Sikkim, and Mizoram etc., which are geographically closer to countries like China, Nepal, and Bhutan (1,15,27). However, very limited biological and genomic data are available on the diversity of the bacterial populations among the different geographical origins of India. In light of the success in differentiating '*Ca. L. asiaticus*' populations at locus CLIBASIA_01645 (6), the present study was directed to determine genetic diversity among the populations of '*Ca. L. asiaticus*' infecting various citrus cultivars in different geographical locations of India based on the tandem repeat (AGACACA) number variability in the same genomic locus.

MATERIALS AND METHODS

Sample collection and culture maintenance. Surveys were conducted between the years 2010 and 2013 to record the incidence and distribution of HLB in different geographical regions of India (Fig. 1). We collected potential HLB symptomatic twigs, leaves, and bud sticks from different citrus cultivars from 12 states (Table 1; Fig. 1). Representative samples were used for biological indexing by graft inoculation on Mosambi sweet orange (*Citrus sinensis*)

indicator plants and were maintained in an insect-proof greenhouse at temperatures of 30°C during the day and 25°C at night.

DNA extraction and '*Ca. L. asiaticus*' detection. The collected symptomatic samples were washed, wiped with 70% ethanol to avoid any surface contamination, and blot dried. Midribs and petioles were excised and ground in liquid nitrogen, and 100 mg of the powdered sample was used for DNA extraction using the DNeasy Plant mini kit (Qiagen, Hilden, Germany) according to the manufacturer's protocol. '*Ca. L. asiaticus*' in these samples was identified using previously described polymerase chain reaction (PCR) amplification of 16S rDNA primer set OI1/OI2c (14,21).

PCR amplification for bacterial population analysis. The PCR amplification targeting CLIBASIA_01645 locus was carried out using primers LapGP-1f (5' GACATTTCACGGTATCGAC 3') and LapGP-1r (5' GCGACATAATCTCACTCCTT 3') (6). The reaction was carried out in a 25 µl reaction volume containing 100 ng of total DNA, 1× PCR buffer, 1.5 mM MgCl₂, 0.2 mM of dNTP each, 0.4 mM of each primer, 1.25 U of GoTaq flexi DNA polymerase (Promega) and nuclease-free water. The My Cycler thermal cycler (Bio-Rad, Hercules, CA) was programmed for one cycle of 4 min at 95°C followed by 35 cycles of 30 s at 95°C, 45 s at 58°C, 60 s at 72°C, and a final extension at 72°C for 10 min. The amplified DNA was visualized in a UV GelDoc system (Bio-Rad) after electrophoresis with ethidium bromide at a constant voltage of 100 V for 90 min in a 1.5% agarose in 1× TAE buffer.

Nucleotide sequencing and data analysis. Amplified PCR products were excised and eluted from the gel using the GenElute Gel Extraction Kit (Sigma-Aldrich, St. Louis, MO) and sequenced (Chromous Biotech Pvt. Ltd., Bangalore, India) using the ABI BigDye v 3.1 terminator cycle sequencing protocols (Applied Biosystems, Foster City, CA). Fluorescent labeled extension products were analyzed by the dye terminator method on an ABI 3500xL DNA sequencer (Applied Bio-systems). Sequence similarity searches were conducted using the BLAST algorithm of the National Center for Biotechnology Information (NCBI) and visual FASTA result tool of the European Molecular Biology Laboratory, European Bioinformatics Institute (EMBL-EBI). Tandem repeats (AGACACA) present in the CLIBASIA_01645 genomic locus were counted and compared manually. Similarly, the frequencies of different TRNs among genotypes were calculated and bacterial population differences were statistically analyzed by an F test (16).

RESULTS

Disease incidence, symptoms, transmission, and culture maintenance. Out of 151 samples collected from different states representing different geographical regions (Fig. 1), a total of 81 samples were positive for HLB with OI1/OI2c set of primers. Among them, 38 were mandarins (*Citrus reticulata*), 19 were sweet orange (*Citrus sinensis*), 12 were acid lime (*Citrus aurantifolia*), three were pomelo (*Citrus grandis*), two of each were sour orange (*Citrus aurantium*), Assam lemon (*Citrus limon*) and Rangpur lime (*Citrus limonia*), and one each were of Cadena Fina (*Citrus sinensis* 'Cadenera'), Dancy tangerine (*Citrus tangerine*), and grape fruit (*Citrus paradisi*) (Table 1). A comparison of the disease incidence among different states of India showed that 47% of the samples from northern Maharashtra, 62% from eastern Maharashtra (Vidarbha region), 30% from Madhya Pradesh, 71% from Uttar Pradesh, 50% from Uttarakhand, 73% from Tamil Nadu, 26% from Andhra Pradesh, 41% from Karnataka, 70% from West Bengal, 80% from Sikkim, 37% from Mizoram, and 72% of the samples from Assam were found infected. During the present survey, all samples collected from Manipur were diagnosed as negative for HLB. However, more extensive sampling needs to be carried out to ascertain this observation. Predominant symptoms of HLB included prominent yellowing of leaf veins in mandarin, leaf yellowing with green islands and leaf mottling in sweet orange, chlorotic leaves and yellow mottling in acid lime, pomelo, and grapefruit, and general yellowing

of leaf in Rangpur lime and Assam lemon. Upon graft inoculation, the disease was successfully transferred to indicator plants with characteristic HLB symptoms within 3 to 6 months postinoculation. All HLB-affected seedlings were maintained in an insect-proof greenhouse for further study.

Analysis of the CLIBASIA_01645 locus. Samples that were PCR-positive with the primer set OI1/OI2c also showed positive amplification with the primer set LapGP-1f/LapGP-1r and generated an approximate amplicon of 650 bp (Fig. 2A) with single PCR amplicon group (SAG). However, in some cases, variations in the amplicon size potentially reflecting the TRN variation were observed (Fig. 2B). All these amplified products were sequenced from both orientations and sequence details have been deposited in the GenBank database under accession nos. KJ885187 to KJ885255 and KJ921625 to KJ921636. Homology searches using the BLAST tool at the NCBI database revealed significant sequence similarities with the CLIBASIA_01645 locus of '*Ca. L. asiaticus*' strain psy62. Sequence analysis showed tandem repeats of AGACACA found in all amplicon sequences (Table 1); however, TRNs varied among the

isolates. In Maharashtra state, especially in Vidarbha region, '*Ca. L. asiaticus*' strains with TRNs of 9 (TRN9) were predominant (Table 1) with a frequency of 35%. Except for one isolate from Assam, Maharashtra was the only state in which the TRN9 strain was observed (Table 1). In the northeastern states of India, especially in Assam, the bacterial strain with TRNs of 7 (TRN7) was predominant with a frequency of 40.6%. The strain with the highest TRNs (TRN17) was reported in the sweet orange cultivar Mosambi from the northern Maharashtra region (Supplementary Tables S1 and S2).

As the TRNs among isolates from most of the geographical regions were variable, we classified the genotypic variation based on the number of TRNs. We categorized the strains into four classes; Class I of TRNs ≤ 5 , Class II of TRNs > 5 but ≤ 10 , Class III of TRNs > 10 but ≤ 15 , and Class IV of TRNs > 15 . We observed that the Class II genotype was most predominant (54.32%) followed by the Class I genotype (30.86%), and the least predominant was Class IV genotype (2.46%). Maharashtra state, particularly the northern Maharashtra region, was the only state with Class IV genotype (Fig. 1, Table 1). '*Ca. L. asiaticus*' populations from the eastern

Fig. 1. Map of India showing the distributions of Indian '*Candidatus Liberibacter asiaticus*' isolates collected from different citrus growing regions of India. The classification is based on the varying tandem repeat numbers (TRNs) present at the CLIBASIA_01645 genomic locus. All Indian '*Ca. L. asiaticus*' isolates are classified under four classes (classes I to IV, refer to the main manuscript for further details) and shown on the map with the help of different shapes. The name of the states from where samples were collected are mentioned on the map and other states are left blank for clarity.

state of West Bengal and the northeastern states of Sikkim and Mizoram were of Class II (71.8%), and in Assam nearly 95% of the bacterial populations were of Class II (Supplementary Fig. S1). In northern Maharashtra, Uttar Pradesh, Tamil Nadu, Karnataka, Sikkim, and Assam, Class III genotype was observed with an overall frequency of 12.34%, while Andhra Pradesh and Uttarakhand predominately had Class I genotype (Fig. 3). The results of test of significance with an F test showed significant differences ($P = 0.05$) for the distribution of frequencies of TRNs in '*Ca. L. asiaticus*' isolates between the states.

In an effort to study association of citrus cultivars with specific classes of the pathogen, DNA was isolated from different citrus cultivars infected with '*Ca. L. asiaticus*' and the CLIBASIA_01645 locus was analyzed. Among mandarin, Class II genotype was more predominant with a 68.42% frequency, followed by Class I and Class III with frequencies of 28.94 and 2.63%, respectively (Fig. 4). The sweet orange predominately contained Class II genotype with 52.63%. However, sweet orange was the only group that also contained Class IV genotype. In contrast, acid limes and Rangpur lime predominately contained Class I and Class III genotype, respectively, and the remaining cultivars contained varying amounts of Class I and Class II genotype (Fig. 4). The analysis of variance test (F test) among the distribution of TRNs within different citrus cultivars was observed as nonsignificant (at $P = 0.05$).

DISCUSSION

India, with diverse environmental conditions in different states, would potentially support increased pathogen diversity in the country. Knowledge of the genetic diversity of '*Ca. L. asiaticus*' from different geographical regions and citrus cultivars is required for predicting the disease risk. Although HLB has been reported in India to infect all commercial cultivars for many years, the study of genomic variations among the '*Ca. L. asiaticus*' bacterial population has not been available. The primer set LapGP-1f/LapGP-1r for CLIBASIA_01645 locus has been used successfully to characterize '*Ca. L. asiaticus*' populations from China and the United States (6,26). Chen et al. (6) observed single PCR amplicon group (SAG), whereas Ma et al. (26) observed both SAG and multiple PCR amplicon group (MAG) using the same set of primers, i.e., LapGP-1f/LapGP-1r for CLIBASIA_01645 locus. We used a similar approach to characterize the CLIBASIA_01645 locus to study the bacterial populations from different citrus growing regions in India and observed presence of SAG (Fig. 2) as observed with '*Ca. L. asiaticus*' populations from China and the United States (6).

DNA sequences with tandem repeats are known to be highly dynamic in both prokaryote and eukaryote genomes. Based on the TRN copy number variation, Chen et al. (6) classified the Florida and Guangdong bacterial populations into two strain groups: TRN

TABLE 1. Tandem repeat numbers (TRN) in CLIBASIA_01645 locus of '*Candidatus Liberibacter asiaticus*' population from different states and citrus hosts grown in India^a

Sample	State/region	Host		Isolate code	Accession no.	TRN	Classification
		Cultivar	Botanical name				
1	Northern Maharashtra, Western India	Nagpur mandarin	<i>Citrus reticulata</i>	'MH-R-CM'	KJ885230	8	Class II
2	Northern Maharashtra, Western India	Mosambi, sweet orange	<i>Citrus sinensis</i>	'MH-R-MO'	KJ885231	9	Class II
3	Northern Maharashtra, Western India	Rangpur lime	<i>Citrus limonia</i>	'MH-R-RL'	KJ885232	12	Class III
4	Northern Maharashtra, Western India	Acid lime	<i>Citrus aurantifolia</i>	'MH-R-AL'	KJ885233	9	Class II
5	Northern Maharashtra, Western India	Mosambi, sweet orange	<i>Citrus sinensis</i>	'MH-BL3P4'	KJ885234	7	Class II
6	Northern Maharashtra, Western India	Mosambi, sweet orange	<i>Citrus sinensis</i>	'MH-BL3P7'	KJ885235	13	Class III
7	Northern Maharashtra, Western India	Mosambi, sweet orange	<i>Citrus sinensis</i>	'MH-BL3P21'	KJ885236	17	Class IV
8	Northern Maharashtra, Western India	Mosambi, sweet orange	<i>Citrus sinensis</i>	'MH-BL3P25'	KJ885237	2	Class I
9	Northern Maharashtra, Western India	Mosambi, sweet orange	<i>Citrus sinensis</i>	'MH-BL4P11'	KJ885238	12	Class III
10	Northern Maharashtra, Western India	Mosambi, sweet orange	<i>Citrus sinensis</i>	'MH-BL5P5'	KJ885239	16	Class IV
11	Vidarbha-Maharashtra, Central India	Nagpur mandarin	<i>Citrus reticulata</i>	'IPB-24'	KJ885209	9	Class II
12	Vidarbha-Maharashtra, Central India	Nagpur mandarin	<i>Citrus reticulata</i>	'IPB-49'	KJ885210	9	Class II
13	Vidarbha-Maharashtra, Central India	Nagpur mandarin	<i>Citrus reticulata</i>	'IPB-66'	KJ885211	9	Class II
14	Vidarbha-Maharashtra, Central India	Nagpur mandarin	<i>Citrus reticulata</i>	'IPB-118'	KJ885212	5	Class I
15	Vidarbha-Maharashtra, Central India	Nagpur mandarin	<i>Citrus reticulata</i>	'IPB-127'	KJ885213	8	Class II
16	Vidarbha-Maharashtra, Central India	Nagpur mandarin	<i>Citrus reticulata</i>	'B35-7'	KJ885214	9	Class II
17	Vidarbha-Maharashtra, Central India	Nagpur mandarin	<i>Citrus reticulata</i>	'B35-10'	KJ885215	5	Class I
18	Vidarbha-Maharashtra, Central India	Nagpur mandarin	<i>Citrus reticulata</i>	'B35-13'	KJ885216	8	Class II
19	Vidarbha-Maharashtra, Central India	Nagpur mandarin	<i>Citrus reticulata</i>	'B35-51'	KJ885217	5	Class I
20	Vidarbha-Maharashtra, Central India	Nagpur mandarin	<i>Citrus reticulata</i>	'B35-120'	KJ885218	9	Class II
21	Madhya Pradesh, Central India	Nagpur mandarin	<i>Citrus reticulata</i>	'R4P9'	KJ885224	5	Class I
22	Madhya Pradesh, Central India	Nagpur mandarin	<i>Citrus reticulata</i>	'Ex-1'	KJ885225	6	Class II
23	Madhya Pradesh, Central India	Nagpur mandarin	<i>Citrus reticulata</i>	'Ex-2'	KJ885226	6	Class II
24	Uttar Pradesh, North India	Mosambi, sweet orange	<i>Citrus sinensis</i>	'A-3'	KJ921632	6	Class II
25	Uttar Pradesh, North India	Mosambi, sweet orange	<i>Citrus sinensis</i>	'An'	KJ921633	13	Class III
26	Uttar Pradesh, North India	Mosambi, sweet orange	<i>Citrus sinensis</i>	'AN-3'	KJ921634	6	Class II
27	Uttar Pradesh, North India	Mosambi, sweet orange	<i>Citrus sinensis</i>	'AN-4'	KJ921635	6	Class II
28	Uttar Pradesh, North India	Mosambi, sweet orange	<i>Citrus sinensis</i>	'AN-5'	KJ921636	5	Class I
29	Uttarakhand, North India	Pummelo	<i>Citrus grandis</i>	'P-3'	KJ885255	4	Class I
30	Tamil Nadu, South India	Mangudi acid lime	<i>Citrus aurantifolia</i>	'TNmal-2'	KJ885244	5	Class I
31	Tamil Nadu, South India	Acid lime	<i>Citrus aurantifolia</i>	'TNal-3'	KJ885245	13	Class III
32	Tamil Nadu, South India	Acid lime	<i>Citrus aurantifolia</i>	'TNal-4'	KJ885246	4	Class I
33	Tamil Nadu, South India	Acid lime	<i>Citrus aurantifolia</i>	'TNal-5'	KJ885247	11	Class III
34	Tamil Nadu, South India	Acid lime	<i>Citrus aurantifolia</i>	'TNal-6'	KJ885248	10	Class II
35	Tamil Nadu, South India	Acid lime	<i>Citrus aurantifolia</i>	'TNal-7'	KJ885249	5	Class I
36	Tamil Nadu, South India	Acid lime	<i>Citrus aurantifolia</i>	'TNal-8'	KJ885250	5	Class I
37	Tamil Nadu, South India	Acid lime	<i>Citrus aurantifolia</i>	'TNal-9'	KJ885251	8	Class II
38	Tamil Nadu, South India	Acid lime	<i>Citrus aurantifolia</i>	'TNal-10'	KJ885252	7	Class II
39	Tamil Nadu, South India	Acid lime	<i>Citrus aurantifolia</i>	'TNal-11'	KJ885253	5	Class I
40	Tamil Nadu, South India	Acid lime	<i>Citrus aurantifolia</i>	'TNal-12'	KJ885254	13	Class III

(continued on next page)

^a Class I (TRN ≤ 5), Class II (TRN > 5 ≤ 10), Class III (TRN > 10 ≤ 15), and Class IV (TRN > 15).

copy less than 10 (TRN < 10) or more than 10 (TRN > 10), but we observed that this scheme was inadequate to accommodate much diversity seen with Indian ‘*Ca. L. asiaticus*’ strains. In ‘*Ca. L. asiaticus*’ populations from Florida, TRN5 was the predominate genotype with a frequency of 84.4%, followed by TRN4 with much smaller frequency of 4.5%, whereas in ‘*Ca. L. asiaticus*’ populations of Guangdong, TRN7 was the most frequent (47.6%), followed by TRN6 with a frequency of 29.2% (6) and TRN 9, 10, 11, and 12 were not observed or nonexistent (6). On the contrary, Indian ‘*Ca. L. asiaticus*’ populations with TRN numbers 9, 10, 11, 12, and 13 (a total of 24.4%) were widely represented. ‘*Ca. L. asiaticus*’ populations with TRN2 and TRN17 were also observed but were not recorded in ‘*Ca. L. asiaticus*’ populations of China and the United States (6). Therefore, to accommodate a more diverse TRN pattern/genotype in a simplified manner, we arbitrarily classified the Indian ‘*Ca. L. asiaticus*’ populations as Class I to IV.

The Maharashtra state has the greatest acreage of citrus cultivation and is a large producer of citrus in India. This state also has a long history of citrus cultivation and sweet orange is a widely grown citrus cultivar. Our results reflect that the ‘*Ca. L. asiaticus*’ strains collected from Maharashtra state belonged to all four classes, suggesting higher variability compared with other citrus growing states. Class II of ‘*Ca. L. asiaticus*’ was observed in all states except Andhra Pradesh, which potentially suggests selection pressure (unknown biotic- or abiotic-factors) for genotypes as was earlier reported by Chen et al. (6) for Florida and Guangdong populations of ‘*Ca. L. asiaticus*’.

Fig. 2. Gel picture showing the polymerase chain reaction bands, which were amplified using the primers set OI1/OI2c. **A**, Primer pair LapGP-1f/LapGP-1r was used and obtained a fragment size of 1.2 kb shown in Lanes 1 to 8. Lane number 9 was used as a negative control. In lane L, 1-kb size marker was used. **B**, Variation in fragment size is visible owing to the variation of tandem repeat copy numbers at the CLIBASIA_01645 locus. In lane L, the size marker of 100 bp was used.

TABLE 1. (continued from preceding page)

Sample	State/region	Host		Isolate code	Accession no.	TRN	Classification
		Cultivar	Botanical name				
41	Andhra Pradesh, South India	Cadenera fine	<i>Citrus sinensis</i> ‘Cadenera’	‘AP6’	KJ885187	5	Class I
42	Andhra Pradesh, South India	Madam venus	<i>Citrus sinensis</i>	‘AP9’	KJ885188	5	Class I
43	Andhra Pradesh, South India	Dancy Tangerine	<i>Citrus tangerine</i>	‘AP2’	KJ885189	5	Class I
44	Andhra Pradesh, South India	Sour orange	<i>Citrus aurantium</i>	‘AP7’	KJ885190	5	Class I
45	Karnataka, South India	Pummelo	<i>Citrus grandis</i>	‘CH-R1’	KJ885219	11	Class III
46	Karnataka, South India	Sour orange	<i>Citrus aurantium</i>	‘CH-S3’	KJ885220	3	Class I
47	Karnataka, South India	Grapefruit	<i>Citrus paradise</i>	‘CH-G4’	KJ885221	10	Class II
48	Karnataka, South India	Coorg mandarin	<i>Citrus reticulata</i>	‘CH-CR5’	KJ885222	5	Class I
49	Karnataka, South India	Coorg mandarin	<i>Citrus reticulata</i>	‘CH-U6’	KJ885223	6	Class II
50	West Bengal, Eastern India	Pummelo	<i>Citrus grandis</i>	‘Kal-3’	KJ921625	7	Class II
51	West Bengal, Eastern India	Khasi mandarin	<i>Citrus reticulata</i>	‘Kal-4’	KJ921626	6	Class II
52	West Bengal, Eastern India	Khasi mandarin	<i>Citrus reticulata</i>	‘Kal-5’	KJ921627	5	Class I
53	West Bengal, Eastern India	Khasi mandarin	<i>Citrus reticulata</i>	‘Kal-10a’	KJ921628	4	Class I
54	West Bengal, Eastern India	Khasi mandarin	<i>Citrus reticulata</i>	‘Kal-10b’	KJ921629	4	Class I
55	West Bengal, Eastern India	Khasi mandarin	<i>Citrus reticulata</i>	‘Kal-12a’	KJ921630	5	Class I
56	West Bengal, Eastern India	Khasi mandarin	<i>Citrus reticulata</i>	‘Kal-12b’	KJ921631	8	Class II
57	Assam, Northeast India	Khasi mandarin	<i>Citrus reticulata</i>	‘KhM-ii’	KJ885191	7	Class II
58	Assam, Northeast India	Khasi mandarin	<i>Citrus reticulata</i>	‘KhM-iii’	KJ885192	7	Class II
59	Assam, Northeast India	Mosambi, sweet orange	<i>Citrus sinensis</i>	‘Mo-i’	KJ885193	8	Class II
60	Assam, Northeast India	Assam lemon	<i>Citrus limon</i> L. Burmf	‘AsL-i’	KJ885194	7	Class II
61	Assam, Northeast India	Khasi mandarin	<i>Citrus reticulata</i>	‘KhM-i’	KJ885195	9	Class II
62	Assam, Northeast India	Khasi mandarin	<i>Citrus reticulata</i>	‘KhM-vii’	KJ885196	6	Class II
63	Assam, Northeast India	Khasi mandarin	<i>Citrus reticulata</i>	‘KhM-vi’	KJ885197	7	Class II
64	Assam, Northeast India	Khasi mandarin	<i>Citrus reticulata</i>	‘AK2-KhM’	KJ885198	8	Class II
65	Assam, Northeast India	Khasi mandarin	<i>Citrus reticulata</i>	‘AK3-KhM’	KJ885199	8	Class II
66	Assam, Northeast India	Khasi mandarin	<i>Citrus reticulata</i>	‘AK4-KhM’	KJ885200	7	Class II
67	Assam, Northeast India	Khasi mandarin	<i>Citrus reticulata</i>	‘AK5-KhM’	KJ885201	7	Class II
68	Assam, Northeast India	Khasi mandarin	<i>Citrus reticulata</i>	‘AK7-KhM’	KJ885202	7	Class II
69	Assam, Northeast India	Khasi mandarin	<i>Citrus reticulata</i>	‘AK8-KhM’	KJ885203	7	Class II
70	Assam, Northeast India	Mosambi, sweet orange	<i>Citrus sinensis</i>	‘AK9-Mo’	KJ885204	7	Class II
71	Assam, Northeast India	Mosambi, sweet orange	<i>Citrus sinensis</i>	‘AK10-Mo’	KJ885205	7	Class II
72	Assam, Northeast India	Mosambi, sweet orange	<i>Citrus sinensis</i>	‘AK11-Mo’	KJ885206	13	Class III
73	Assam, Northeast India	Mosambi, sweet orange	<i>Citrus sinensis</i>	‘AK12-Mo’	KJ885207	7	Class II
74	Assam, Northeast India	Mosambi, sweet orange	<i>Citrus sinensis</i>	‘AK13-Mo’	KJ885208	6	Class II
75	Mizoram, Northeast India	Khasi mandarin	<i>Citrus reticulata</i>	‘M-5’	KJ885227	7	Class II
76	Mizoram, Northeast India	Assam lemon	<i>Citrus limon</i> L. Burmf	‘M-2’	KJ885228	4	Class I
77	Mizoram, Northeast India	Pummelo	<i>Citrus grandis</i>	‘M-4’	KJ885229	8	Class II
78	Sikkim, Northeast India	Sikkim mandarin	<i>Citrus reticulata</i>	‘SK-6’	KJ885240	6	Class II
79	Sikkim, Northeast India	Sikkim mandarin	<i>Citrus reticulata</i>	‘SK-13’	KJ885241	13	Class III
80	Sikkim, Northeast India	Sikkim mandarin	<i>Citrus reticulata</i>	‘SK-14’	KJ885242	5	Class I
81	Sikkim, Northeast India	Sikkim mandarin	<i>Citrus reticulata</i>	‘SK-16’	KJ885243	5	Class I

Although the primary reason for TRN copy number variation among the '*Ca. L. asiaticus*' isolates is unknown, it is widely believed that it provides the selective advantages to the bacteria adapting to newer set of environmental conditions such as host species, soil, and temperature variations (25,26). Both biotic and abiotic factors are known to play a significant role in affecting the repeat number instability; examples include CT repeat contraction

upon fungal infection (29) and repeats in bacteria that undergo frequent adaptive, environmentally stimulated mutations (28). In the present study, we noticed that isolates with a similar TRN copy numbers were not favored by any particular citrus cultivar. However, the higher TRN copy numbers (TRN > 10 but ≤ 15) were only found among widely grown citrus cultivars, mandarin, sweet orange, and acid lime. We also noticed that '*Ca. L. asiaticus*'

Fig. 3. Percent distribution of '*Candidatus Liberibacter asiaticus*' isolates collected from different states of India that were surveyed during this study. The class II isolate is predominantly higher in all states followed by class I and then class III isolates. The class IV isolate is localized and found only in the Maharashtra state, which has the greatest area coverage under citrus crops in India.

Fig. 4. Percent distribution of Indian '*Candidatus Liberibacter asiaticus*' types in various citrus cultivars. Note that the most important commercial cultivars grown in India are mandarin, sweet orange, and acid lime and also the only cultivars that support all three '*Ca. L. asiaticus*' strains. The star indicates that a single sample was analyzed in this study under these cultivars.

genotypes with highest TRN (>15) only found in the state of Maharashtra in sweet orange might suggest adaptive selection of the pathogen. Similar incidences of high TRN copy numbers in 'Ca. L. asiaticus' isolates were localized to the central part of Florida (6), although pathogen adaptability was not suggested.

In the northeastern states of India, 'Ca. L. asiaticus' genotypes TRN6 and TRN7 were predominant (53.12%) and this area is geographically closer to Guangdong region of China where predominant presence of TRN6 and TRN7 genotypes were observed (6). This region, with its long history of citrus cultivation and believed as the center of citrus origin, perhaps lead to the localized stabilization of genotypes having similar TRNs.

In summary, analysis of the TRNs in the CLIBASIA_01645 locus of 'Ca. L. asiaticus' associated with the HLB of citrus suggests that India represents a much more diversified set of 'Ca. L. asiaticus' populations than previously reported for south China and Florida. The CLIBASIA_01645 locus of the 'Ca. L. asiaticus' was found to be useful to detect and characterize the genomic variability of the bacterial population. This study represents the first attempt to document variations of Indian 'Ca. L. asiaticus' strains and potentially would help in understanding the variabilities of global 'Ca. L. asiaticus' populations.

ACKNOWLEDGMENTS

We thank T. Schafer, The Whitney Laboratory for Marine Bioscience, University of Florida, for critically reviewing the manuscript and D. K. Agrawal, Principal Scientist, ICAR-DSR, and U. Pradesh for performing statistical analysis.

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