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Assessment and molecular detection of *Candidatus* Liberibacter *asiaticus* in mandarin orange and acid lime in Tamil Nadu

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ABSTRACT: The Greening disease of citrus, popularly known as Huanglongbing (HLB), has been severe in India since late 1966. It is associated with infection by '*Candidatus* Liberibacter asiaticus,' a heat-sensitive, non-cultured, phloem-limited alpha-proteobacterium. Leaf samples exhibiting greening symptoms were picked up from 26 Mandarin orange trees from each 5 orchards in the Dindigul district and 13 Acid lime trees from each 5 farms of Nilgiris, Dindigul and salem districts of Tamil Nadu. Among ten amplicons were derived from nine samples. Nine samples among them yielded between 1, 100-bp to 1160bp amplicon indicative of '*Ca.* L. asiaticus' infection, the mandarin orange samples are amplified with A2 and J5 primers which showed 690bp amplicon size targeting (rplKAJL-rpoB) Beta operon gene of Las. The amplicons obtained from the samples were sequenced, and all showed approximately 1 160bp, identical to the cognate '*Ca.* L. asiaticus' NCBI GenBank. Based on the survey results, the phylogenetic tree was built and interpreted. It has been concluded that up to date, only '*Ca.* L. asiaticus' is the only strain associated with citrus greening (HLB) in commercial citrus Orchards of Tamil Nadu.

Keywords: HLB, Mandarin orange, Acid lime, *Candidatus* Liberibacter asiaticus, Singleplex PCR

INTRODUCTION

Mandarin orange, *Citrus reticulata* L. and acid lime, *Citrus aurantifolia* (Christm) Swingle (Family: Rutaceae) are the most popular and widely grown citrus fruits worldwide. Citrus production is affected by several biotic stress factors, namely bacteria, fungi, viruses, and nematodes. Among the bacterial diseases, citrus greening disease Huanglongbing disease is the most destructive disease that declines citrus yield in India and other countries of Asia, the Pacific, and Africa (Ahlawat, 1997; Bove, 2006; Lee, 1921; Mccleane*t al*., 1965). The symptoms of HLB on the leaves of infected citrus trees range from complete yellowing, asymmetrical blotchy mottling, and fruits with these symptoms have a small size, asymmetrical shape, inverted colour, aborted seeds, poor flavour, and excessive fruit drop (Anon, 1996; Lin 1956; Tolba and Soliman, 2015). In India, Huanglongbing, caused by *Candidatus* Liberibacter asiaticus (Varma *et al*., 1993), is one of India's most dangerous citrus diseases. HLB has a high negative impact on the yield of mandarin plants grown in warm, humid regions. The disease is caused by Gram-negative fastidious bacterium (Garnier *et al*., 1984), *Candidatus* Liberibacter asiaticus in Asia, *Candidatus* Liberibacter africanus in Africa (Jagoueix *et al*., 1994), and *Candidatus* Liberibacter americanus in South America (Teixeira *et al*., 2005).

The HLB is a phloem-limited, non-culturable alpha proteobacterium (Jagoueix *et al.,* 1994). The term "*Candidatus*" in Latin binomial indicates that the bacterium is not culturable in an axenic medium and is only characterized by molecular DNA-based techniques (Murray and Schleifer, 1994). Fraser and Singh (1968) reported that the decline in citrus production in Punjab was due to the Presence of Huanglongbing disease. In India, Dr Lilian R. Fraser first reported the citrus Huanglongbing in 1966 (Fraser *et al*., 1966). It was after that reported in different citrus-growing regions of Bihar, West Bengal, and Sikkim (Nariani and Raychaudhuri, 1968). Thus it would be very significant to detect the HLB-causing organism in mandarin orange orchards in south India and better manage them appropriately. Detecting this fastidious bacterium is difficult because of its non-culturability, low concentration, and uneven distribution in its natural hosts (Su and hang 1974; Graca 1991). The disease is diagnosed by biological indexing on indicator hosts (Nariani and Raychaudhuri, 1968), which is time-consuming, and symptoms depend on temperature. The disease, therefore, cannot be diagnosed easily by conventional procedures such as electron microscopic examination of ultrathin sections and bioassay on indicator plants. As a substitute, a reliable and rapid detection protocol by PCR was developed, giving quick results for early detection of HLB.

MATERIALS AND METHODS

Survey for huanglongbing disease at Various citrus growing groves of Tamil Nadu

A rapid roving survey was conducted in different citrus-growing areas of Dindugal and Thirunelveli, Tamil Nadu, from March 2018 to March 2019. During the surveys, symptom expressions on different citrus species

A

Lane1- TMOL1
Lane2- TMOL2

Fig. 1 A. Genomic DNA isolated from different samples of HLB affected mandarin orange plants. *TMOL1- Kanalkadu mandarin orange midrib Farm1 *TMOL2- Thandikudi mandarin orange midrib farm 2

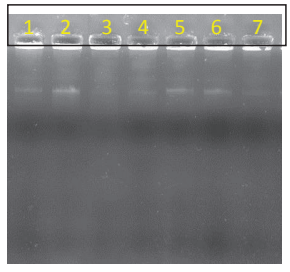
were noticed. Symptomatic samples presumed to have HLB infection were amassed and immediately stored in a cool box to avoid DNA degradation. The amassed samples were brought to the laboratory and processed promptly or stored for DNA extraction at -20°C or -80°C for future use.

Detection of CLas bacterium associated with huanglongbing disease of citrus in Tamil Nadu

Total DNA was extracted from the leaf lamina and midribs separately from the collected samples using Plant DNeasy mini kit (Qiagen, Germany). DNA was isolated following two validated protocols, DNeasy Plant Mini Kit (Qiagen, Germany) or CTAB extraction protocol. The CTAB method gave a good yield of DNA but a low quality compared to the QiagenDNeasy Plant Mini Kit. DNA isolation using the DNeasy Plant Mini kit was done following the manufacturer's protocol using a column for DNA isolation. For DNA extraction, 100 mg of samples were used. The quantity and quality of the isolated DNA were determined by taking OD values at 260nm and 280nm using a NanoDrop Spectrophotometer (NanoDrop, TNAU).

PCR amplification: Amplification was executed in Thermal cycler (Eurofins) through conventional PCR using primers set OI1F/OI2cR (Ahmad and sijam, 2009) targeting partial 16SrDNA (most specific region of the CLas genome). The reaction mixture was geared up for 25µl volume using 0.5µl of dNTPs (10mM),5µl of 10x buffers and 2.0µl MgCl2(25mM), 2.0µl of forward and reverse primers (10µM), DNA template of 5µl (100-200 ng/µl) and 0.3µl Taq polymerase (5 units/µl, Genei TM), and remaining volume was makeup with nuclease-free water. The thermal cycle conditions were: one cycle at95ºC for two minutes, followed by 35 cycles at 95ºC

B



Lane1- SALL1
Lane2- SALL2
Lane3- SALL3
Lane4- SALL4
Lane5- SALM1
Lane6-SALM2
Lane7-SALM3

*SALL-survey acid lime leaf
*SALM-survey acid lime midrib

Fig. 1 B. Genomic DNA isolated from different samples of HLB affected Acid lime plants

for 40 seconds, followed by 60ºC for one minute and 72ºC one minute, followed by a 72ºC extension for 10 min(Ahmad and sijam, 2009). The amplification product was examined at 1% agarose gel containing ethidium bromide in Tris-acetate EDTA buffer. The amplicons were looked over through UV illumination in a gel documentation system.

Phylogenetic analyses

Sequences retrieved in this work and all the 16S rRNA nucleotide sequences of '*Ca.* Liberibacter asiaticus available in the NCBI database (Table 1, 2, and Table 3) were aligned by using ClustalW software. For phylogenetic analyses, 16S rRNA gene sequences of all known '*Ca.* Liberibacter asiaticus' and related representative strains were analyzed by minimum evolution analysis using the Neighbor-Joining method and bootstrap analyzed by MEGA6.06 software (http://www.megasoftware.net/ index. HTML) (Tamura *et al.*, 2007).

RESULTS AND DISCUSSION

Huanglongbing disease survey at different citrus growing pockets of Tamil Nadu

Most of the samples were collected during the warmer season (March-April) of the year as the concentration of CLas bacterium was expected to be more during these periods. Throughout the survey, diverse symptoms varying from yellowing of leaves, and branches, rabbit ear-like appearance of leaves, irregular mottling on leaf lamina (blotchy mottle shoot with yellow patches), mineral deficiencies like symptoms (a regular pattern of yellowing or vein yellowing or clearing on leaf lamina) were observed. Interestingly, leaves with mineral deficiency-like symptoms and rabbit ear-like appearance

Fig 2A. Gel picture ofOi1 and Oi2c Primers PCR amplified mandarin orange midrib samples showing 1160 base pairs similar to *Candidatus* Liberibacter asiaticus.1160 base pairs similar to *Candidatus* Liberibacter asiaticus

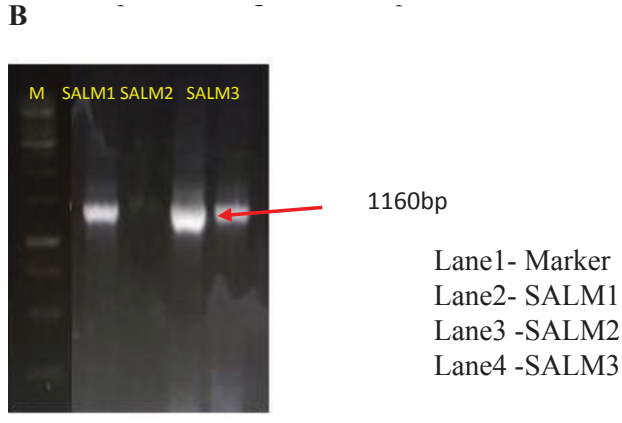


Fig 2B. Gel picture of Oi1 and Oi2c Primers PCR amplified Acid lime MID RIB samples showing 1160 base pairs similar to *Candidatus* Liberibacter asiaticus

were found positive for CLas. The irregular blotching called mottling and chlorosis is the peculiar symptoms of HLB (Baranwal, 2004). These determinations indicated that CLas isolates prevalent in Tamil Nadu exhibit different symptoms under field conditions. For example, all the citrus species collected from different screened areas were sensitive to HLB, as described earlier (Garnier and Bove, 1993; Garnier and Bove, 1994a). The contrasting symptoms observed under field conditions in the present study might be due to the pervasiveness of various strains/haplotypes of *Candidatus* Liberibacter asiaticus bacterium and susceptibility patterns of the citrus genotypes reported by Tsai *et al.* (2008).

CLas bacterium detection associated with huanglongbing disease of citrus in Tamil Nadu

The CLas bacterium was detected by PCR employing the primer targeting partial 16S rDNA (the most conserved region of the CLas bacterium genome). The expected amplicon of approx. 1160 bp was observed on the agarose gel in the citrus samples having CLas infections. Out of the fourteen samples collected from different locations of citrus growing pockets of Tamil Nadu, thirteen samples tested positive (90.00 per cent infection). The details of the samples, symptoms expression at the field level and host plant are given in Table 2. The single band of intact genomic DNA was visualized on the agarose gel (Fig. 1A, 1B, 2A, 2B and 2C).

PCR DETECTION

The 16S r RNA region of these bacterial isolates was amplified with universal primer pair FD1 and RP2 primer, and PCR fragments with the size of 1500 bp and 16s r DNA Las gene identifying OI1 and Oi2c specific primers were used. PCR fragments with the size of 1160bp were obtained respectively (Fig. 2B). Based on the 16s ribosomal sequence analysis, *Candidatus* Liberibacter asiaticus was identified in mandarin orange samples number1 and sample 2 from Thandikudi and kanalkadu field and Acid lime samples collected from various districts such as Dindigul, yercaud, sankarankoil and kallar districts which are samples 1,2,3 and sample number 4. The sequence has been submitted in NCBI GenBank and acquired the following accession numbers such as OP832019 and OP895022 amplified at 690bp by A2 and J5 primer set and MT671371 for Mandarin Orange amplified at 1100bp by Oi1 and Oi2c primer set. The sequence JQ867421.1 was taken from the NCBI database to compare Mandarin isolate and native isolates. OP895023 and OP895024 amplified at 690bp by A2 and J5 primer set and Accession numbers – MT671445, MT671446, MT671447, and MT671448. For Acid Lime Orange amplified at 1100bp by Oi1 and Oi2c primer set. MT671371 is the accession number obtained from the DNA sequence of the Mandarin orange midribs, whereas MT671445, MT671446, MT671447, and MT671448 are DNA sequences obtained from the Acid lime leaf and Midribs. All the samples were obtained through midribs. Interestingly leaf samples without midrib are also showed positive to CLas for acid lime samples (Table. 1)

Phylogeny analysis

The detected strains were compared with the other *Candidatus* Liberibacter asiaticus spp. ID in parenthesis Such as AB008366.1, KY990821.1,KU761591.1, JQ867432.1, DQ303210.1, AB038369.1, KY211974.1, LN795908.1, KY230624.1, KY008940.1, MK142766.1, AB008366.1, DQ303210.1,

C

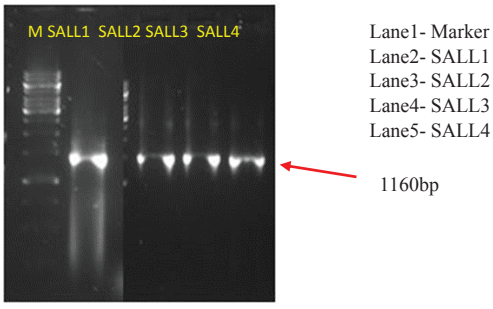


Fig. 2C. Gel picture of Oi1 and Oi2c Primers PCR amplified Acid lime LEAF samples showing 1160 base pairs similar to *Candidatus* Liberibacter asiaticus

D

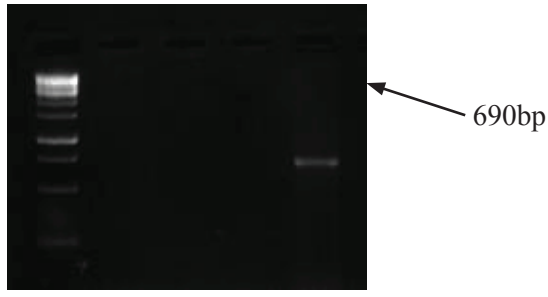


Fig. 2D. Gel picture of A2 and J5 Primers PCR amplified mandarin orange midrib sample showing 1160 base pairs similar to *Candidatus* Liberibacter asiaticus

KY008940.1, and also with the different strains of Candidatus genus, which were enormously infecting other continents such as North America and South Africa, are as follows KF170062.1(*Ca*. L. Solanacearum), L22533.1 (*Ca*. L. Africanus), AY742824.1 (*Ca*. L. americanum).

Interpretation of Phylogenetic analysis

The 16S rRNA amplicons obtained from nine representatives HLB affected citrus plants were sequenced using Specific Primers. The 1167 bp long sequences shared 100% nucleotide identity, indicating a low polymorphism level among strains from the same geographical region. The sequences had 99% sequence identity with previously reported nucleotide sequences of '*Ca.*Liberibacter asiaticus', confirming the PCR data and showed 100%sequence identity with '*Ca.* Liberibacter Americans, 78% with '*Ca.*Liberibacter Africanus-related isolates 75% with '*Ca.* Liberibacter solanacearum'. Minimum evolution phylogenetic analysis of 16S rRNA gene sequences revealed that '*Ca.* Liberibacter asiaticus isolates from Tamil Nadu clustered together in a phylogenetic subclade with known '*Ca.* Liberibacter asiaticus isolates (Fig. 3). Simultaneously, the 16sRNA sequences of 9 samples which were showing consistent positive result to CLas through PCR were compared with the NCBI retrieved sequences and they were correlated with each other. The similarity between the retrieved and isolated samples were 100% and the similarity between acid lime and mandarin orange isoalted samples is 88 percent.

The *Candidatus Liberibacter asiaticus* strains showed 99% similarity with the clade of CLas-affected *Diaphorina citri* nucleotide sequence, which was acquired from NCBI GenBank (AB038369.1). Liberibacter endosymbiont of *Diaphorina citri* gene, which gave evidence of Asia citrus psyllid (*Diaphorina citri*), was a perfect vector for transmission of greening disease from plant to plant.

***Sample Names:**

TMOL1 =*Candidatus* Liberibacter asiaticus Farm1 (Mandarin orange)

Assessment and prevalence of Las in Collected samples

The prevalence was observed for the present study; 12 samples were collected from 10 farms at various locations of citrus growing pockets of Tamil Nadu; 11 samples tested positive; the total samples showed positive for *Candidatus* Liberibacter asiaticus (90%). The samples from Sankarankoil, Ayyampalayam and kallar showed positive to both leaf and midribs. (Table. 1)

Assessment and prevalence of Las in Collected places

The characteristics of the samples, symptoms expression at the field level, and host plant are given in Table 2. Percentage infection of HLB was highest at Dindigul, sankarankoil and kallar districts (100%), followed by Salem district (75%), which was the lowest per cent infection was recorded amongst the samples

CONCLUSION

The survey has given a complete understanding of the prevalence of HLB occurrence in the citrus belts of Tamil Nadu. The PCR-based assay has overcome difficulties caused by the low concentration and uneven distribution of HLB in citrus. The diagnosis of HLB by PCR was excellent and essential for screening diseased plants and establishing disease-free citrus nurseries. Although the cost of PCR and DNA hybridization methods is higher than that of an immunological assay such as ELISA, it can provide reliable data quickly. HLB detection using PCR should facilitate epidemiological studies, aiding in HLB control. These findings are being used extensively

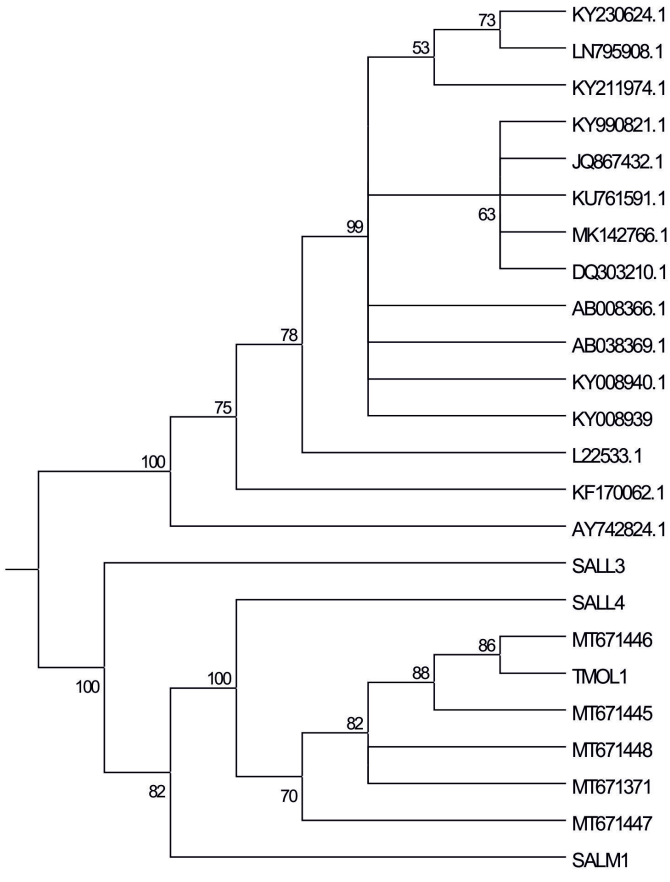


Fig 3. A. Phylogenetic experiments were carried out using 16S-rDNA sequences of Candidatus Liberibacter asiaticus MT6711371, MT671445, MT671446, MT671447, and MT671448 with 16S-rDNA sequences of other Candidatus Liberibacter asiaticus spp. ID in parenthesis indicates the GenBank number referring to the 16S-rDNA sequence of the corresponding organisms.

Table 1. List of leaves and midribs showing positive for Las extracted from Mandarin and Acid lime samples

Place	Crop	Samples	Farm No	Part extracted		*Candidatus* Liberibacter asiaticus Positive/negative
				leaf	midrib	
Thandikudi	Mandarin orange	TMOL1	1	-	midrb	Positive
Kanalkadu	Mandarin orange	TMOL2	2	-	midrb	Positive
Yercaud	Acid lime	SALL1	1	-	midrb	Positive
Sankaran Koil	Acid lime	SALL2/SALL3	2	leaves	midrb	Positive
Kallar	Acid lime	SALL3/SALM3	3	leaves	midrb	Positive
Ayyampalayam	Acid lime	SALL4/SALM4	4	leaves	midrb	Positive

*Total nine samples showed positivity

*Two samples are midribs of mandarin orange

* Four samples are midribs of acid lime, three samples are leaves of acid lime

Table 2. Pervasivenss of CLas bacterium based on 16S rDNA of both Acid lime and Mandarin orange

Sl.no.	Districts				No. of the sample tested positive for HLB	Incidence of HLB (%) *
		Acid lime	Mandarin orange	Total		
1	Dindigul	2	2	4	4	100
2	Yercaud	4		4	3	75
3	Sankarankoil	2		2	2	100
4	Kallar	2		2	2	100
Total	**four**			**12**	**11**	**90**

*PCR based detection

for HLB diagnosis in Tamil Nadu's citrus belts and help supply HLB-free seedlings to registered nurseries for multiplication in huge numbers.

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