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DETECTION AND MOLECULAR CHARACTERIZATION OF *CANDIDATUS LIBERIBACTER* SPP. CAUSING HUANGLONGBING (HLB) IN INDIGENOUS CITRUS CULTIVARS IN PAKISTAN

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Abstract

Citrus greening or huanglongbing (HLB) is one of major devastating citrus diseases all over the world. This disease is caused by fastidious α-proteobacterium, *Candidatus liberibacter* spp. and is transmitted by grafting as well as psyllids *Diaphorina citri* or *Trioza erytreae*. The objective of this study was to identify and characterize the huanglongbing (HLB) infectious pathogen in commercial (Kinnow and sweet oranges) varieties by using molecular markers such as 16S rRNA, 16S/23S rRNA and outer membrane protein fragments from symptomatic leaves of assorted citrus varieties. DNA extracted from forty different citrus (including mandarin and sweet oranges) varieties having HLB-symptomatic plants from different orchards of Pakistan. Gene-specific primers for 16SrDNA, 16S/23S rDNA and outer membrane protein (OMP) gene regions were used for identification of *Ca. liberibacter* spp. An amplified fragment of 1174 bp from 16SrDNA, 900 bp of 16S/23S rRNA and 600 bp was observed for OMP gene fragments of Asian isolates. The resulted fragments were TA cloned and sequenced from both strands. The infectious bacterium was identified as *Candidatus liberibacter asiaticus* and was found in 17 samples (42%). The seasonal variation on prevalence of *Candidatus liberibacter asiaticus* in citrus varieties was well observed. It declined during spring season due to unfavourable temperature and humidity for *Candidatus liberibacter asiaticus* because disease symptoms showed mostly at low humidity and warm temperature (up to 35°C).

Key words: Huanglongbing, *Candidatus liberibacter asiaticus*, Outer membrane protein, *Diaphorina citri*, α-proteobacterium.

Introduction

Huanglongbing (HLB) is one of the most destructive citrus diseases, previously known as Citrus greening. In Asia HLB has damaged probably 60 million citrus trees. Major symptoms of HLB disease on susceptible trees shows blotchy mottling leaves, yellowish shoots, chlorosis and twig dieback soon after. Trees are shorten in height, reduced growth, lop-sided and small-sized fruits that are imperfectly green in colour. HLB disease is difficult to detect because some of infected trees symptoms are confused with Zn deficiency (Tatineni *et al.*, 2008). Huanglongbing (HLB) is mainly distressing disease with no any effective control. It is caused by nonculturable bacterium designated *Candidatus liberibacter* that is a gram-negative and phloem-limited fastidious agent. The HLB-associated bacterium *Candidatus liberibacter* is transmitted by grafting and vectors named psyllids *Diaphorina citri* and *Trioza erytreae*. Three pathogenic species i.e., *Candidatus liberibacter asiaticus*, *Candidatus liberibacter africanus*, and *Candidatus liberibacter americanus* are recognized by using 16S rDNA sequence (Bove, 2006) and *Candidatus liberibacter asiaticus* is the more prevalent species (e.g., Asia, Brazil, and North America).

HLB infectious agent is widespread throughout Pakistan for many decades, this disease is found in all neighbouring countries. HLB pathogen occurs in almost all provinces of Pakistan i.e., Sindh, KPK, Punjab and Kashmir. During surveys Akhtar (1999) reported Citrus greening disease prevalence in Kinnow (22%), sweet orange (25–40%), grapefruit (15%) and in sweet lime(10%). Inconsistency in disease cruelty and prevalence have been observed at assorted locations by morphological and symptomatic studies. However, the HLB disease occurs at higher rate nearby in Peshawar and Punjab (about 35 %).

HLB associated infectious pathogen is difficult to detect due to its nonspecific nature of disease symptoms as it occurs at low titer in host tissues. It is also complicated to control because it transmitted by vector and vegetative propagation. HLB disease has prolonged latency period and may remain symptomless in certain host plants. These symptomless infected plants prove more hazardous as they are continuous source of infection spread through vector. HLB bacterial disease symptoms are also similar with nutrient (Zn) deficiency which lead to yellowing shoots of plants (Weinert *et al.*, 2004).

Huanglongbing (HLB) or Citrus greening is the most caustic bacterial disease in viable citrus cultivars. All major commercial citrus species and varieties are at risk. There is no any successful treatment is known apart from avoid the HLB infectious plants or the removal of infected trees. As it has appeared a latent threat to the citrus production a strong molecular evidence are required to examine the HLB associated infectious pathogen in citrus.

Keeping all these factors in view, the objectives of this study was to detect and compared the HLB bacteria in different varieties of citrus with HLB symptoms in different orchards and determination of prevalent *spp.* of *Candidatus liberibacter* in various geographic regions of Pakistan and effect of seasonal variations on its prevalence. Molecular markers give positive results for detection, phylogenetic and evolutionary relationship among bacteria and other prokaryotes and genetic characterization of *C. liberibacter* (Nageswara *et al.*, 2013). Several molecular targets such as 16S/23S rRNA intergenic spacer region, 16S rRNA gene and *OMP* gene fragments were used to characterize different isolates of *C. liberibacter*.

Materials and Methods

Sample collection: Huanglongbing (HLB) symptomatic leaves samples were collected from the trees showing yellowish shoots, blotchy mottling leaves and upright branching pattern. Almost 35 varieties of citrus (Kinnow mandarin, Fewtrell's, Mosambi and other sweet oranges) were obtained from orchard of "Citrus research institute, Sargodha, Pakistan" in winter (Jan-Feb), spring (March-April), summer (June, Jul) and Fall (Oct-Nov) to check the prevalence of HLB in different seasons. For each sample, at least 50 symptomatic leaves were collected in labeled polythene bags and transferred to ice box until dispatched to the laboratory.

DNA extraction: Total DNA of Citrus plants was extracted using protocol described by Murray & Thompson, 1980 with some modification (Naz *et al.,* 2013). Two hundreds mg midrib tissues from fresh leaves was taken and grinded in liquid nitrogen. After that 3 ml of CTAB (Cetyl Trimethylammonium Bromide) extraction buffer [100 mMTris–HCl pH 8.0, 20 mM EDTA (Ethylenediaminetetraacetic acid), 1.4 M NaCl, 2% (wv-1) 2-mercaptoethanol 2%] was added and incubated for 30 min at 65 °C. Samples were centrifuged at 6000 rpm for 10 min and then transferred the supernatant into new eppendorf. Equal volume of chloroform/ isoamylalcohol (24:1) was added in that collected supernatant. Then washed the DNA pellet with 70% ethanol and resuspended in 100 µl of TE buffer. The DNA extracts were stored at –20°C after quantification by taking OD_{260} in spectrophotometer (Naz *et al.,* 2015).

PCR Amplification: Conventional PCR was used to identify the occurrence of HLB associated bacterium in citrus by amplification of 16S rDNA conserved region. PCR was carried out 25 µl total volume of reaction mixture containing 10X buffer, 25mM MgCl2, 0.2 mM of dNTPs 25ng of DNA template, 0.4 µM of both primer reverse and forward (Table 1) and Taq DNA used 0.75 units. DNA amplification was done on advanced Primus-96 Thermal cycler and cycling condition included pre-PCR denaturation at 95°C for 2 min, followed by 35 cycles at 95°C for 40 sec, 58°C for 30 sec and 72°C for 1 min followed final extension at 72°C for 10 min. For 16S/23S rRNA cycling parameters were: pre-PCR denaturation at 95°C for 2 min, followed by 35 cycles at 95°C for 40 sec, 58°C for 30 sec and 72°C for 1 min followed final extension at 72°C for 10 min And for OMP gene fragments amplification PCR was performed as pre-PCR denaturation at 95°C for 2 min, followed by 35 cycles at 95°C for 40 sec, 58°C for 30 sec and 72°C for 1 min followed final extension at 72°C for 10 min The amplified PCR products were analyzed by electrophoresis on 1% agarose gels (1× TAE buffer) run at 110 volt for 30 min after staining with ethidium bromide and then under UV illumination visualized the gel.

DNA cloning and sequence analysis: The expected PCR products were purified using Thermo Scientific Gene JET PCR Purification Kit (Lot # 00129583) according to manufacturer's procedure, ligated in InsT/A clone™ PCR Product Cloning Kit (PCR 2.1 vector) from Invitrogen Life Technologies and transferred in DH5α strain of *E. coli*. Recombinant plasmids were confirmed by colony PCR and verified the presence of desired insert of the expected size by restriction analysis. Randomly chosen clones from infected varieties were sequenced using ABI Prism DNA sequencer (Perkin-Elmer, USA). The consensus sequences were compared and analyzed by with those of 16S rDNA region obtained from Genbank data bases. The final sequence was submitted to EMBL database.

Results

Detection and Molecular characterization of HLB associated pathogen, *C. liberibacter*, was carried out in indigenous citrus cultivars in Pakistan using 16S rDNA, 16S/23S rRNA and *OMP* gene fragments.

Amplification of 16S rDNA, 16S/23S rRNA and *OMP* gene fragments: PCR with gene targeted primers were used for detection of *C liberibacter* from different orchards of Pakistan. PCR products of 1174 bp from 16S rDNA, 900 bp from 16S/23S rDNA intergenic region sequence and 600, 600 and 1040 bp for *OMP* gene fragments of *C. liberibacter* were obtained in HLB-diseased leaves of different citrus cultivars. Forty different citrus (including mandarin and sweet oranges) varieties having HLB-symptomatic plants from different orchards of Pakistan were analyzed (Table 2). Although the plants showed classic HLB symptoms, on the first PCR survey using the16S rDNA primers, the bacterium was detected in 17 samples analyzed (42%) in which an amplified fragment of 1174 bp of 16S rDNA was generated (Fig. 1) and with16S/23S rRNA 900 bp size was amplified (Figs. 1 & 2). The outer membrane protein (*OMP*) gene fragments of the Asian isolates were amplified by three sets of primer pairs i.e., *OMP* fragment 1, *OMP* fragment 2 and *OMP* fragment 3. First two primer pairs yielded PCR products of 600 bp in size but *OMP* fragment 3 primer pair generated about 1040 bp product (Fig. 2).

Table 1. Nucleotide sequences of Primers pair for polymerase chain reaction (PCR).

Primers	Nucleotide sequences	Product size (bp)
16S rDNA	5'-GCGCGTATGCAATACGAGCGGCA-3' 5'-GCGTCGCGACTTCGCAACCCAT-3'	1174
16S/23S rRNA	5'-ATGGGTTGCGAAGTCGCGAGGC-3' 5'-CGCCCTTC TCTCGCGCTTGA-3'	900
OMP fragment 1	5'-ACACCATTCTATTTCTCCGA-3' 5'-TTAATTGGATCTCCCTCACTC-3'	600
OMP fragment 2	5'-AAAGCGGATAGAAATTGAGG-3' 5'-GATATCAACATGCCTTTACGTG-3'	600
OMP fragment 3	5'-GCGGGCCTGTTGATAATCCTGC-3' 5'-CAACAACCCTGACCTCCATC-3'	1,040

Table 2. Detection of *Candidatus Liberibacter* by using different molecular markers.

Sr. No.	Name of plants	Molecular markers		
		16S rDNA	16S/23S rRNA	OMP gene fragments
1.	Kinnow	+	+	+
2.	Musambi	+	+	+
3.	Jaffa	+	+	+
4.	Pine Apple	-	-	-
5.	Netal	+	+	+
6.	Ruby sweet	+	+	+
7.	OlindaValencia	-	-	-
8.	Washington navel	-	-	-
9.	Moro blood	+	+	+
10.	Tracco nucellar	-	-	-
11.	Kozan	+	+	+
12.	Pera Rio	+	+	+
13.	Hamlin	+	+	+
14.	Navelina	-	-	-
15.	Robel	-	-	-
16.	Rangpur lime	-	-	-
17.	Dancy	-	-	-
18.	New hall	+	+	+
19.	Valancia Late	+	+	+
20.	Mars early	-	-	-
21.	Salustiana	-	-	-
22.	Tarocco	-	-	-
23.	Emby gold	-	-	-
24.	Campbell Valencia	+	+	+
25.	Parson Brown	-	-	-
26.	Casa grand	+	+	+
27.	Lane Navel	-	-	-
28.	Spring Navel	-	-	-
29.	Frost Red	-	-	-
30.	Navelate	+	+	+
31.	Succari	-	-	-
32.	Sangnello	-	-	-
33.	Ruby blood	+	+	+
34.	Hinkley	-	-	-
35.	Glene Navel	-	-	-
36.	Olinda Valencia	-	-	-
37.	Mid sweet	+	+	+
38.	Westin	-	-	-
39.	Robble	-	-	-
40.	Eureka Lemon	+	+	+

The amplicons were sequenced from both strands, and consensus sequences were submitted to EMBL DNA database under accession numbers LN835770. The 16SrDNA sequence identified the infectious agent was *C. liberibacter asiaticus* that was 99% similar to sequence of the same region of isolate from Malaysia (Accession No. EU224393). The sequence of 16S/23S rDNA of *C. liberibacter asiaticus* sp. shared 99% homology with unculturable bacteria isloate from mudflat sediment (Accession No. FR870068.1). The sequence of OMP gene fragments of indigenous *C. liberibacter asiaticus* sp., shared 95% homology with unculturable bacteria isolate from wheat (Accession No. HE662556.1).

A phylogenetic tree generated by Clustal W**,** displaying the 16S rDNA sequence of Pakistani Kinnow isolate showed 99% nucleotide similarity with *C. liberibacter asiaticus* strain (Fig. 3). In this study kinnow isolate with 16S rDNA gene of *C. liberibacter asiaticus* from Florida and Malaysian strains showed 99% nucleotide similarity. These sequences of 16S rDNA of Kinnow isolate confirmed that the infectious agent was *C. liberibacter asiaticus*.

The seasonal variation on prevalence of *C. liberibacter asiaticus* population in citrus varieties was also observed (Table 3). It declined during spring season (March, April and May) due to unfavourable temperature and humidity for *C. liberibacter asiaticus* beacuse disease symptoms showed mostly at low humidity and warm temperature (up to 35°C) While in the winter (December, January and Feburary) *C. liberibacter* population was comparatively less in citrus plants. In the Summer and Fall season when the average maximum temperatures were 35 to 40°C, a higher number of *C. liberibacter* was observed in different citrus varieties. This result indicates during months that are not favorable for detection of *C. liberibacter asiaticus* from the psyllid, the bacterium is detectable from the leaf tissue.

Discussion

The occurrence of huanglongbing (HLB) citrus greening bacterium *C. liberibacter asiaticus* in various citrus orchards of Pakistan was perceived in this study. *C. liberibacter asiaticus* spp. was detected in 17 citrus varieties showing distinctive HLB symptoms. HLB susceptible plants mostly remains symptomless and has long incubation period in certain host plants and these infectious plants occur in fields as reported by Weiner *et al.* (2004). Using the set of primers targeting 16S rDNA, 16S/23S rDNA and *OMP* gene fragments (Table 1) HLB associated bacteria *C. L. asiaticus* was detected in samples from various citrus varieties. These gene specific primers amplified desired fragments of rRNA intergenic region and the outer membrane protein fragments of *C. liberibacters* from citrus leaf midrib samples (Fig. 2). While amplification in healthy leaves was not showed which has been used as negative control (Remziye *et al.*, 2014).

Forty different citrus varieties having HLB-symptomatic plants from different orchards of Pakistan were analyzed the bacterium was detected in 17 samples in the initial PCR survey using the 16S rDNA primers. An amplified fragment of 1174 bp of 16S rDNA was generated. The infectious agent HLB bacteria has been identified in Africa as *C. liberibacter africanus* and in Asia has been detected as *C. liberibacter asiaticus* by PCR amplification with same 16S rDNA gene specific primer (Teixeira *et al.*, 2005). HLB disease had been confirmed in Malaysia by detection and sequenced of 16S rDNA gene of *C. liberibacter asiaticus*. The sequences of 16S rDNA gene of Malaysian isolates are identical to each other and had close relationship with Chinese *C. liberibacter asiaticus* strains. It showed insignificant difference from those African and American Strains (Khairulmazmi *et al.*, 2008). As results showed the 16S rDNA sequences among Asian isolates of *C. liberibacter* were similar with 99 to 100% identity; whereas, among African isolates, the identity was 98.5%.

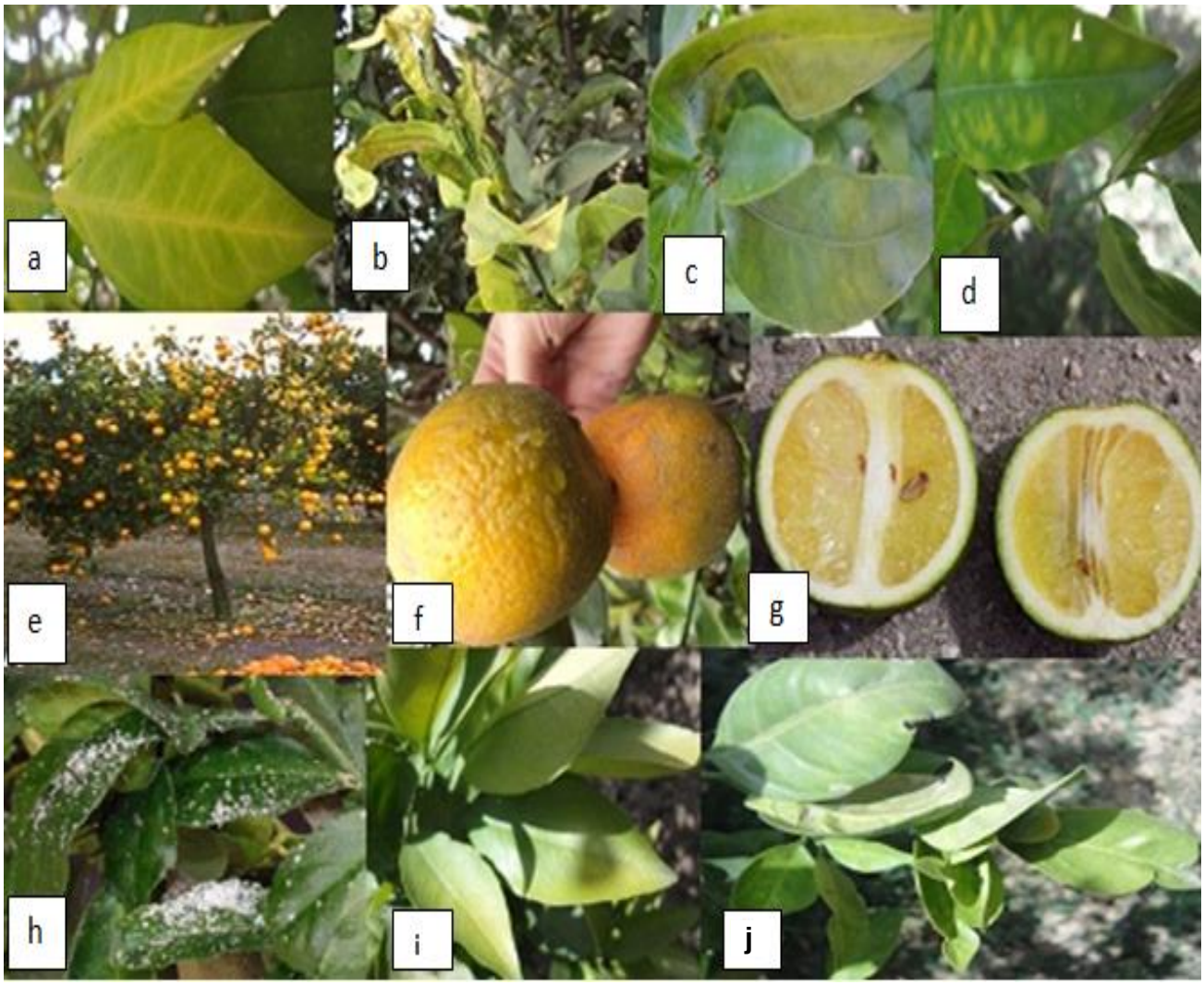

Fig. 1. Symptoms of HLB disease on citrus leaves showing (a)Yellowish vein, (b,c) blotchy and mottling leaves, (d) cholorsis (e) early fruit drop and (f) fruit having green colorations and (g) small, lopsided fruit with aborted seeds (h) honeydew (i) sooty mold (j) notchy leaves.

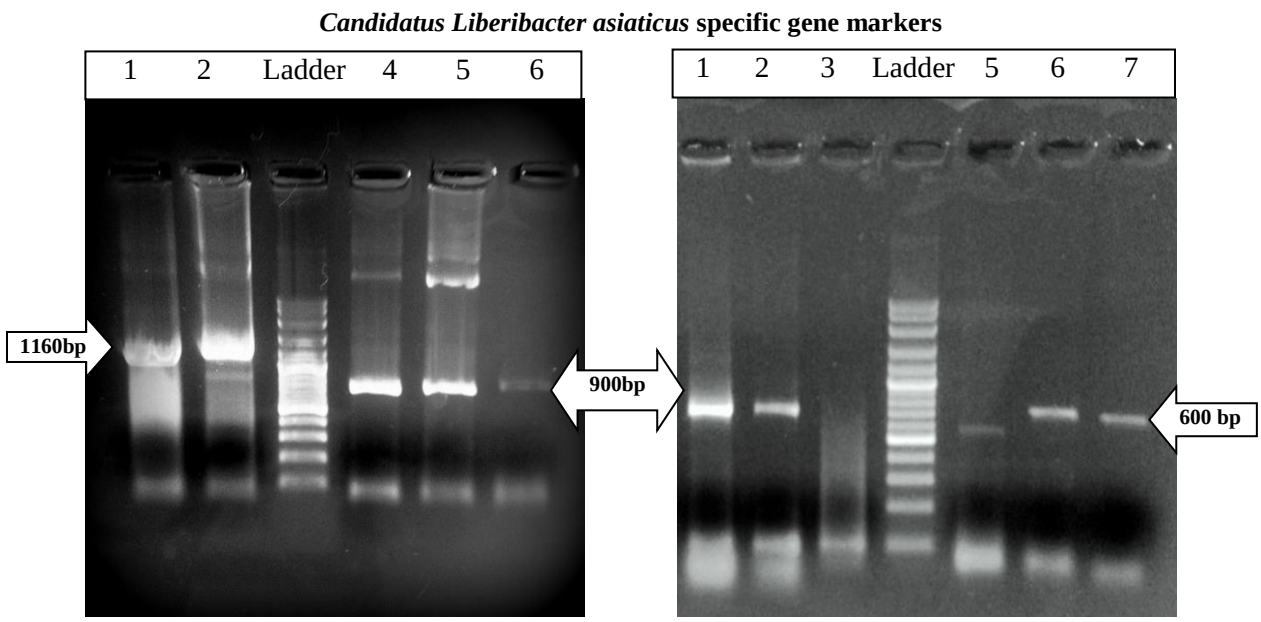

Fig. 2. 1% Agarose gel electrophoresis of DNA amplified with 16S rDNA generated 1174 bp (Lane 1 and lane 2) and 16/23S rRNA primers yielded 900 bp (Lane 4, Lane 5 and lane 6). In Fig. 3. OMP gene fragments amplified (600, 600 and 1040 bp) PCR product in lane 5, lane 6 and lane 7.

Conventional polymerase chain reaction (PCR) was used to amplify the 16S/23S intergenic region (900 bp) of several isolates of citrus greening organism. The resulted sequences of 16S/23S rDNA intergenic region were found similar to reported strains of China and to some extent with Afraican isolates (98.5%) reported by Ding *et al.,* (2009). They also found the Chinese isolates are very close to *C. l. asiaticus* and distinct from *C. l. africanus* and *C. l. americanus.* These results suggest that the Chinese HLB isolates belong to the species *C. liberibacter asiaticus.*

The outer membrane protein gene fragments of the Asian isolates were generated by three sets of primer pairs i.e., *OMP* fragment 1, *OMP* fragment 2 and *OMP* fragment 3. Ahmad *et al.* (2009) also characterized the *Ca. Liberibacter asiaticus* isolates based on outer membrane protein (*OMP*) genes, as it has been showed no significant difference between two isolates. *C. liberibacter asiaticus* isolates had 99% nucleotide similarity and 0.30 the least evolutionary value. The genetic variability of *C. liberibacter asiaticus* strains had also been studied by Bastianel *et al.,* (2005) that *OMP* gene was the most capable gene candidate for examining the inter-and intra-specific isolates diversity.

The seasonal variation effect on the prevalence of bacterium present at high levels in citrus plants in June to August and in October to November. Psyllid counts were very low in both summer and winter. Because the physical conditions are unfavourable for *C. l. asiaticus* during this period, disease symptoms mostly appear at low humidity and warm temperature. Razi *et al.,* 2014 reported that in the winter *C. l. asiaticus* prevalence decreased in citrus plants. Psyllid counts were also very low in both summer and winter. Psyllids showed a high prevalence of *C. liberibacter asiaticus* in spring. This result indicates that June, July and August are the most favorable period for detection studies of *C. liberibacter asiaticus* in citrus plants.

Table 3. Detection of *Candidatus Liberibacter* by using 16S rDNA and 16S/23S rRNA markers at different seasons.

Sr. No.	Name of varieties	Dec/Jan/ Feb		Mar/April/May		Jun/Jul/Aug		Oct/Nov	
		16S rDNA	16S/23S rRNA	16S rDNA	16S/23S rRNA	16S rDNA	16S/23S rRNA	16S rDNA	16S/23S rRNA
1.	Kinnow	+	+	+	+	+	+	+	+
2.	Musambi	-	-	-	-	+	-	-	-
3.	Jaffa	+	+	-	-	+	-	+	+
4.	Pine Apple	-	+	-	+	-	+	-	+
5.	Netal	+	+	+	+	+	+	+	+
6.	Ruby sweet	+	+	+	+	+	+	+	+
7.	Olinda Valencia	-	-	-	-	-	-	-	-
8.	Washington navel	-	+	-	-	+	-	-	-
9.	Moro blood	-	-	-	-	-	+	-	-
10.	Tracco nucellar	-	-	-	-	+	-	+	-
11.	Kozan	+	+	+	+	+	+	+	+
12.	Pera Rio	+	+	+	+	+	+	+	+
13.	Hamlin	+	+	+	+	+	+	+	+
14.	Navelina	-	-	-	+	-	+	-	+
15.	Robel	-	-	-	-	-	-	-	-
16.	Rangpur lime	-	-	-	-	+	+	-	-
17.	Dancy	-	-	-	-	-	-	-	-
18.	New hall	-	+	-	+	-	+	-	+
19.	Valancia Late	-	+	-	+	+	+	-	-
20.	Marrs early	-	-	-	+	+	+	-	+
21.	Salustiana	-	-	-	-	+	-	-	-
22.	Tarocco	-	-	-	-	-	-	-	-
23.	Emby gold	-	-	-	-	+	+	+	+
24.	Campbell Valencia	-	+	-	-	+	+	-	+
25.	Parson Brown	-	-	-	-	-	-	-	-
26.	Casa grande	-	+	+	+	+	+	-	+
27.	Lane Navel	-	+	-	-	+	-	-	+
28.	Spring Navel	-	+	-	-	-	+	-	+
29.	Frost Red	-	-	-	-	-	-	-	-
30.	Navelate	-	+	-	-	+	+	+	+
31.	Succari	-	+	-	-	-	-	+	-
32.	Sangnello	-	-	-	-	-	-	-	-
33.	Ruby blood	-	+	+	+	+	+	+	+
34.	Hinkley	-	-	+	-	-	-	-	+
35.	Glene Navel	-	-	-	+	-	-	-	-
36.	Olinda Valencia	-	-	-	-	-	-	-	-
37.	Mid sweet	+	+	-	+	+	+	+	+
38.	Westin	-	-	-	-	-	-	-	+
39.	Robble	-	-	-	-	-	-	-	-
40.	Eureka Lemon	+	+	+	+	+	+	-	+

HLB-suspected leaves in Pakistan

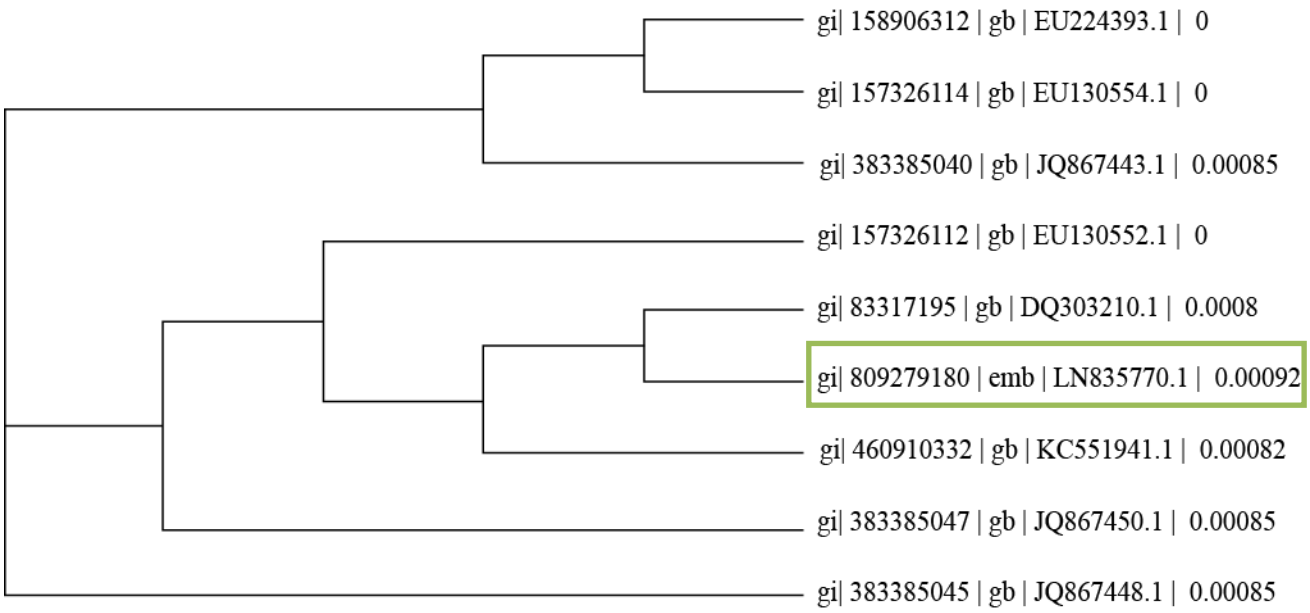

Fig. 3. Phylogenetic tree constructed by Clustal W based on 16S rDNA gene sequences, showing the positions of Kinnow isolate and representatives of *Candidatu liberibacter* spp. taken from in the Genbank.

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