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Research Article

Bacterial pathogens associated with citrus huanglongbing-like symptoms in southern Iran

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Abstract: Huanglongbing (HLB) also known as citrus greening, is a destructive disease of citrus and now, is considered as a new emergence and spread out threat to the Middle East and North Africa (MENA) citrus production. In a survey conducted in southern Iran in 2013-2014, 77 citrus samples exhibiting symptoms of HLB were collected. Single-step and nested polymerase chain reactions (PCR) were employed to determine the presence of the phloem-limited bacterial pathogens '*Candidatus* Liberibacter asiaticus'(CLas), phytoplasma and *Spiroplasma citri*. Both CLas and phytoplasma were detected in HLB-affected citrus trees as co-infection (7.79%) and single infection (10.38% for phytoplasma and 42.85% for CLas). According to the proposed 16S rDNA-based phytoplasma classification scheme, the HLB-associated phytoplasma from this study was a member of peanut witches'broom (16SrII) phytoplasma group. This is the first report of association of a phytoplasma with HLB in sweet lime in the world and first record of association of CLas with sour orange (*Citrus aurantium* L.) and sweet lime in Iran.

Keywords: *'Candidatus* Liberibacter asiaticus', Citrus greening, Phytoplasma, Citrus, Iran

Introduction

Citrus huanglongbing (HLB), also known as citrus greening, is one of the most destructive diseases of citrus which has spread to many parts of the world and is a threat to the citrus production industry (Bové, 2006; Gottwald, 2010) HLB infects almost all citrus cultivars and causes substantial economic losses by decreasing fruit production, shortening the lifespan of the trees (Bové, 2006; Gottwald, 2010; Graca, 1991) and even killing trees. Globally, more than 60 million trees have already been killed by this disease (Das *et al.*, 2007; Halbert and Manjunath, 2004). Until recently, HLB was only associated with the three species of *'Candidatus* Liberibacter' including *'Ca*. L. asiaticus' (CLas), '*Ca*. L. africanus' and '*Ca.* L. americanus' (Bové, 2006; Gottwald, 2010). CLas, the most widespread species, is found in tropical and subtropical areas in Asia, Brazil (Halbert, 2005; Teixeira *et al.*, 2005), and North America (Halbert, 2005); '*Ca*. L. africanus' is mostly restricted to Africa and is sensitive to high temperatures (Planet *et al.*, 1995; Teixeira *et al.*, 2005); and '*Ca.* L. americanus'

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is currently found only in Brazil (Teixeira *et al.*, 2005). Nevertheless, the examination of other possible HLB infections around the world has led to the identification of an additional etiological agent, *Candidatus* Phytoplasma spp. which are alternately identified as phytoplasma strains belonging to '*Ca*. P. phoenicium' (in Brazil) (Chen *et al.*, 2009; Teixeira *et al.*, 2008), '*Ca*. P. asteris' in China(Chen*et al.*, 2009) and Mexico (Arratia-Castro *et al.*, 2014) and '*Ca*. P. aurantifolia' (in China) (Lou *et al.*, 2014). The pathogens ('*Candidatus* Liberibacter spp.') are transmitted by both contaminated bud woods in nurseries and the psyllids, *Trioza erytreae* in Africa (McClean and Oberholzer, 1965) and *Diaphorina citri* in Asia, North and South America (Halbert, 2005).

In Iran, HLB disease was found for the first time in Sistan-Baluchistan and Hormozgan provinces in 2008 (Faghihi *et al.*, 2009). The disease vector, *Diaphorina. citri*, was apparently distributed throughout the two provinces, and CLas positive psyllids were found in most of these regions (Salehi *et al.*, 2012). Understanding the etiology of HLB is critical for managing the disease. Accordingly, in this study, single-step and nested polymerase chain reactions (PCR) were employed to determine the presence of phloem-limited bacterial pathogens, CLas and phytoplasma associated with HLB in southern Iran.

Materials and Methods

Plant materials

Five to ten mature leaves from each citrus tree with HLB- like symptoms were collected and considered as an HLB sample for DNA extraction. All samples were collected between January 2013 and November 2014 (Table 1).

DNA extraction and polymerase chain reaction (PCR) assays

Total DNA was extracted using the cetyltrimethylammonium bromide (CTAB) method as previously described (Zhang *et al.*, 1998).

In all cases, PCR reactions were carried out in 25 µl of reaction mixture containing: 2 µl of template DNA, 2.5 µl of 10 × reaction buffer, 1.6 mM $MgCl_2$, 0.2 mM of each dNTP, 160 nM of each primer, and 2 units of *Taq* DNA polymerase (CinnaGen Inc, Iran).

PCR was carried out with a Bio-Rad thermal cycler (USA) with initial denaturation of 4 min at 94 °C, followed by 35 cycles of 40 s denaturation at 92 °C, 40 s annealing at 55-60 °C (Table 2), extension at 72 °C, and a final extension of 7 min at 72 °C. PCR products were electrophoresed in a 1% agarose gel and visualized by ethidium bromide staining under UV light.

Two step duplex nested PCR assay

Sensitive and simultaneous detection of three destructive phloem-limited, prokaryotic pathogens of citrus: CLas, *'Ca*. Phytoplasma aurantifolia' and *Spiroplasma citri* the causal agents of HLB, witches' broom disease of lime (WBDL) and stubborn disease of citrus, respectively was carried out by multiplex nested PCR assay (Saberi *et al*., 2015). The detection involved a duplex PCR reaction with universal primers of prokaryotic ribosomal 16S rRNA gene (fD1/rP1) (Weisburg *et al.*, 1991) and P58- 6f/4r for *S. citri* (Yokomi *et al.*, 2008) for the first round, followed by primers set Las606/LSS (Fujikawa and Iwanami, 2012) and fU5/rU3 (Lorenz *et al.*, 1995) for CLas and '*Ca*. Phytoplasma spp'., respectively, in the second round. Moreover, the presence of CLas was tested by the expected amplicons 703 bp using CLas specific primers set A2/J5 (Hocquellet *et al.*, 1999) and 1160bp amplified by OI1/OI2C (Jagoueix *et al.*, 1994) (Table 2) and the presence of phytoplasma was tested by an expected ≈800-bp and ≈1250bp DNA amplicon using specific primers sets fU5/rU3 (Lorenz *et al.*, 1995) and R16F2n/R16R2 (1250 bp) (Gundersen and Lee, 1996) nested with primer pair P1/P7 (Smart *et al*. 1996) (Table 2).

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Table 1 Polymerase chain reaction (PCR) detection of '*Candidatus* Liberibacter asiaticus'(CLas) and '*Ca.* Phytoplasma spp'(Cph) in leaf tissue samples collected from different areas in South Iran, 2013-2014.

Province	Date	No. of plants Samples[1]	PCR result[2]					
			Clas+	Cph +	Clas + Cph +	Clas + Cph-	Clas-Cph +	Clas-Cph -
Kerman	October	14 (14)	9	5	4	5	1	4
Sistan & Baluchestan	November	13 (5,6,0, 1,1)	2	7	1	1	6	5
Hormozgan	January	18 (7,3,7, 1)	4	-	-	4	-	14
Sistan & Baluchestan	April	20 (11,4,2,1,0,2)	13	2	1	11	1	7
Kerman	May	12 (12)	5	-	-	5	-	7

1: Numbers in parentheses are sweet orange (*Citrus sinensis* [L.] Osbeck), lime (*Citrus aurantifolia*), tangerine (*C. reticulata* Blanco), sweet lime (*Citrus limonum*), grapefruit (*Citrus paradisi*) and sour orange (*Citrus aurantium* L.), respectively.
2: CLas = '*Candidatus* Liberibacter asiaticus'. The bacterium was identified by nested PCR using primer sets fD1/fD2 (1st round) and OI1/OI2c (2nd round) or by conventional PCR with primer set A2/J5 as detailed in Table 1. Cph = '*Ca.* Phytoplasma spp'. The bacterium was detected by nested PCR using primer sets P1/P7 (1st round) and fU5/rU3 (2nd round).

Table 2 Information about primers used for characterization of '*Candidatus* Liberibacter asiaticus' and *'Ca.* Phytoplasma spp' strains in this study.

Primer set	Sequence (5′ to 3′)	Putative gene	Amplicon size (bp)	Annealing temperature	Reference
fD1/rP1	agagtttgatcctggctcag/ aaggaggtgatccagcc	16S rRNA	≈1,500	56 °C	Weisburg *et al.* (1991)
OI1/OI2c	gcgcgtatgcaatacgagcggca/ gcctcgcgacttcgcaacccat	16S rRNA	1,160	60 °C	Jagoueix *et al*. (1994)
P1/P7	aagagtttgatcctggctcaggatt/ cgtccttcatcggctctt	16S rRNA	≈1,800	58 °C	Smart *et al*. (1996)
R16F2n/R16R2	gaaacgactgctaagactgg/tgacg ggcggtgtgtacaaaccccg	16S rRNA	1250	60 °C	Gundersen and Lee (1996)
fU5/rU3	cggcaatggaggaaact/ ttcagctactctttgtaaca	16S rRNA	882	55 °C	Seemüller *et al.* (1994)
A2/J5	tataaaggttgacctttgagttt/ acaaaagcagaaatagcacgaacaa	*rplA* gene (β-operon)	669-703	60 °C	Hocquellet *et al*. (1999)
P58-6f/P58-4r	gcggacaaattaagtaataaaagagc/ gcacgcatttgccaactaca	P58, a putative adhesion gene	450	56 °C	Yokomi *et al.* (2010)
Las606/LSS	ggagaggtgagtggaattccga/ acccaacatctaggtaaaaacc	16S rRNA	500	55 °C	Fujikawa *et al.* (2012)

DNA sequencing, phylogenetic and virtual RFLP analyses

Five representative HLB-phytoplasma samples; in single infection with phytoplasma spp. (sample N6) as well as in mixed infections with CLas (samples N1, N2, N4 and I200) alongside a rootstock sample with witches' broom symptom in HLB-infected orchard in Kerman province, Narab (sample N8) were assayed for nucleotide sequences with phytoplasma specific primer sets fU5/rU3 and R16F2n/R16R2 nested with primer set P1/P7.

phytoplasma strains N2 and N8 were selected for standard PCR amplification with primer set P1/P7 for 35 cycles. DNA amplicons (≈1, 800 bp) were cloned into pTZ57R/T vector (Fermentas, Lithuania) and sequenced from both directions. The P1/P7 sequence were compared to the current GenBank database using the BLASTn network service available in

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the National Center for Biotechnology Information (NCBI).

Phytoplasma R16F2n/R2 sequences were compared to the references in the GenBank database, using the BLASTn program. Virtual RFLP analysis was performed on the R16F2n/R2 16Sr DNA sequences from phytoplasma isolate N2 (this study), as well as 11 phytoplasmas affiliated to 16SrII group subgroups (Table 3), using *i*PhyClassifier program (Zhao *et al.*, 2013). Each aligned 16S rDNA sequence was digested *in silico* with 17 restriction enzymes (*Alu*I, *BamH*I, *Bfa*I, *BstU*I, *Dra*I, *EcoR*I, *HaeII*I, *Hha*I, *Hinf*I, *Hpa*I, *Hpa*II, *Kpn*I, *Sau3A*I, *Mse*I, *Rsa*I, *Ssp*I, and *Taq*I) which have routinely been used for phytoplasma 16S rDNA RFLP analysis (Lee *et al.*, 1998). The virtual RFLP patterns were compared and a similarity coefficient (F) was calculated for each pair of phytoplasma strains using *i*PhyClassifier program (Zhao *et al.*, 2013).

Multiple sequence alignments were performed using ClustalW tool in CLC sequencer viewer 6 software. Phylogenetic and molecular evolutionary analyses were carried out using neighbor-joining method of MEGA 7.0 software. *Acholeplasma laidlawii* was selected as the out-group to root the tree and the stability of relationships was assessed by bootstraping in 1000 replication.

Results

Distribution of CLas and HLB-phytoplasma

In total, 77 HLB-like symptomatic citrus samples (77 trees) were collected during October 2013 until April 2014 from citrus orchards situated in southern Iran, including Sistan &Balouchistan (33 samples); Kerman (26 samples) and Hormozgan (18 samples) provinces (Table 1). These samples included 48 sweet orange (*Citrus sinensis* [L.] Osbeck), 13 lime (*Citrus aurantifolia*), nine tangerine (*C. reticulata* Blanco), three sweet lime (*Citrus limonum*), one grapefruit (*Citrus paradisi*) and three sour orange (*Citrus aurantium* L.) (Table 1). Collected samples showed different HLB-like symptoms including asymmetric blotchy leaf mottle, yellowing of the leaf veins, yellowing of the leaf and twig, leathery leaves with yellow mottling in the end half, swollen and corky leaf veins, lopsided, twig dieback and yellowing of mid vein and yellowing between the veins (Fig. 1 and Table 3).

Detection of phytoplasma and '*Ca*. L. asiaticus'

Using direct and/or nested PCR with CLas specific primer pairs (Table 2) 38 out of 77 citrus samples from Sistan-Baluchistan (17 samples), Hormozgan (7 samples) and Kerman (14 samples) provinces were positive for Asian form of HLB (Tables 2 and 3).

An amplicon of 703 bp in single-step PCR was obtained with CLas-specific primer pair A2/J5 (Fig. 2) from symptomatic leaves of HLB-diseased citrus species, including sweet orange (samples I5 and I12), lime (samples S4, S7, I14, I15 and I20), sour orange (samples I1, I2, SS1-SS4), tangerine (sampleI16) and sweet lime (sample I3) from Sistan & Baluchestan and sweet orange (samples N1- N4, F5, F6 and J9) from Kerman (Table 3) provinces. To establish if these samples were actually infected, a more sensitive assay as that afforded by nested-PCR was tried. An amplicon of 1160bp in nested PCR assay using primer pairs fD1/rP1(first round) and OI1/OI2C confirmed the positive results obtained by primer pair A2J5 (Table 3). Moreover, results obtained from nested PCR indicated the association of CLas in citrus samples with HLB symptoms, including sweet orange (samples I9 and I11), and sour orange (sample I19) from Sistan & Baluchestan, tangerine (samples Ba5, Ba5, Ba9, Ba12, Ba13 and Ba15) from Hormozgan and sweet orange from Kerman (N5, F3, F4, F7, J1, J4, J6, J12) provinces, which had already been tested negative by single-step PCR employing A2/J5 primer pair. There was no amplification from leaf extracts of healthy citrus and water control in single step and nested PCR assays.

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Table 3 Samples information collected in south of Iran.

Location (Province)	Sampling date	Isolate	Host	Typical symptoms[1]	PCR result[2]		
					Clas-1 (OI1/OI2c)	Clas-2 (A2/J5)	*C*ph
Sistan & Baluchestan	Nov., 2013	S4	Lime	Sy3	+	+	-
		S3	Sweet orange	Sy3	-	-	+
		S5	Lime	Sy3	-	-	+
		S6	Sweet orange	Sy3	-	-	+
		S7	Lime	Sy3	+	+	+
		S8	Lime	Sy4	-	-	+
		S9	Lime	Sy4	-	-	+
		S10	Sweet lime	Sy8	-	-	+
	2014, Apr	I1	Sour orange	Sy1	+	+	-
		I2	Sour orange	Sy1	+	+	-
		I3	Sweet lime	Sy1	+	+	-
		I5	Sweet orange	Sy1	+	+	-
		I9	Sweet orange	Sy5	+	-	-
		I11	Sweet orange	Sy2	+	-	-
		I12	Sweet orange	Sy2	+	+	-
		I14	Lime	Sy3	+	+	-
		I15	Lime	Sy4	+	+	-
		I16	Tangerine	Sy5	+	+	-
		I19	Sour orange	Sy3	+	-	-
		I20	Lime	Sy3	+	+	+
		I21	Lime	Sy3	-	-	+
	2014, Jul.	SS1	Sour orange	Sy1	+	+	-
		SS2	Sour orange	Sy1	+	+	-
		SS3	Sour orange	Sy1	+	+	-
		SS4	Sour orange	Sy1	+	+	-
Hormozgan	2014, Jan.	Ba5	Tangerine	Sy4	+	-	-
		Ba6	Tangerine	Sy4	+	-	-
		Ba9	Lime	Sy3	+	-	-
		Ba12	Tangerine	Sy4	+	-	-
		Ba13	Tangerine	Sy4	+	-	-
		Ba15	Sweet orange	Sy2	+	-	-
Kerman	2013, Oct.	N1	Sweet orange	Sy6	+	+	+
		N2	Sweet orange	Sy6	+	+	+
		N4	Sweet orange	Sy8	+	+	+
		N5	Sweet orange	Sy5	+	-	+
		N6	Sweet orange	Sy9	-	-	+
		N8	Bakraee	WBDL	-	-	+
		F3	Sweet orange	Sy2	+	-	-
		F4	Sweet orange	Sy2	+	-	-
		F5	Sweet orange	Sy7	+	+	-
		F6	Sweet orange	Sy2	+	+	-
		F7	Sweet orange	Sy3	+	-	-
	2014, May	J1	Sweet orange	Sy3	+	-	-
		J4	Sweet orange	Sy2	+	-	-
		J6	Sweet orange	Sy2	+	-	-
		J9	Sweet orange	Sy2	+	+	-
		J12	Sweet orange	Sy3	+	-	-

[1] Symptoms of collected leaf samples: Sy1 = asymmetric blotchy leaf mottle; Sy2 = yellowing of the leaf veins; Sy3 = yellow mottling, thick, leathery leaves; Sy4 = yellowing of the leaf and twig; Sy5= thick, leathery leaves with yellow mottling in the end half ; Sy6 = swollen, and corky leaf veins; Sy7 = lopsided; Sy8 = twig yellowing of mid vein; Sy9 = yellowing between the veins; ASY = a symptomatic leaf of infected tree; WBDL = witches' broom disease of lime.

[2] polymerase chain reaction analyses; positive (+) or negative result (–) of collected samples in south of Iran. Clas = '*Candidatus* Liberibacter asiaticus'. The bacterium was identified by nested polymerase chain reaction analyses (PCR) using primer sets fD1/fD2 (1st round) and OI1/OI2c (2nd round) (Clas-1) or by A2/J5 (Clas-2) as detailed in Table 1; *C*ph = '*Ca.* Phytoplasma spp'. The bacterium was determined by nested PCR using primer sets P1/P7 (1st round) and fU5/fU3 (2nd round) as listed in Table 2; UN: non determined.

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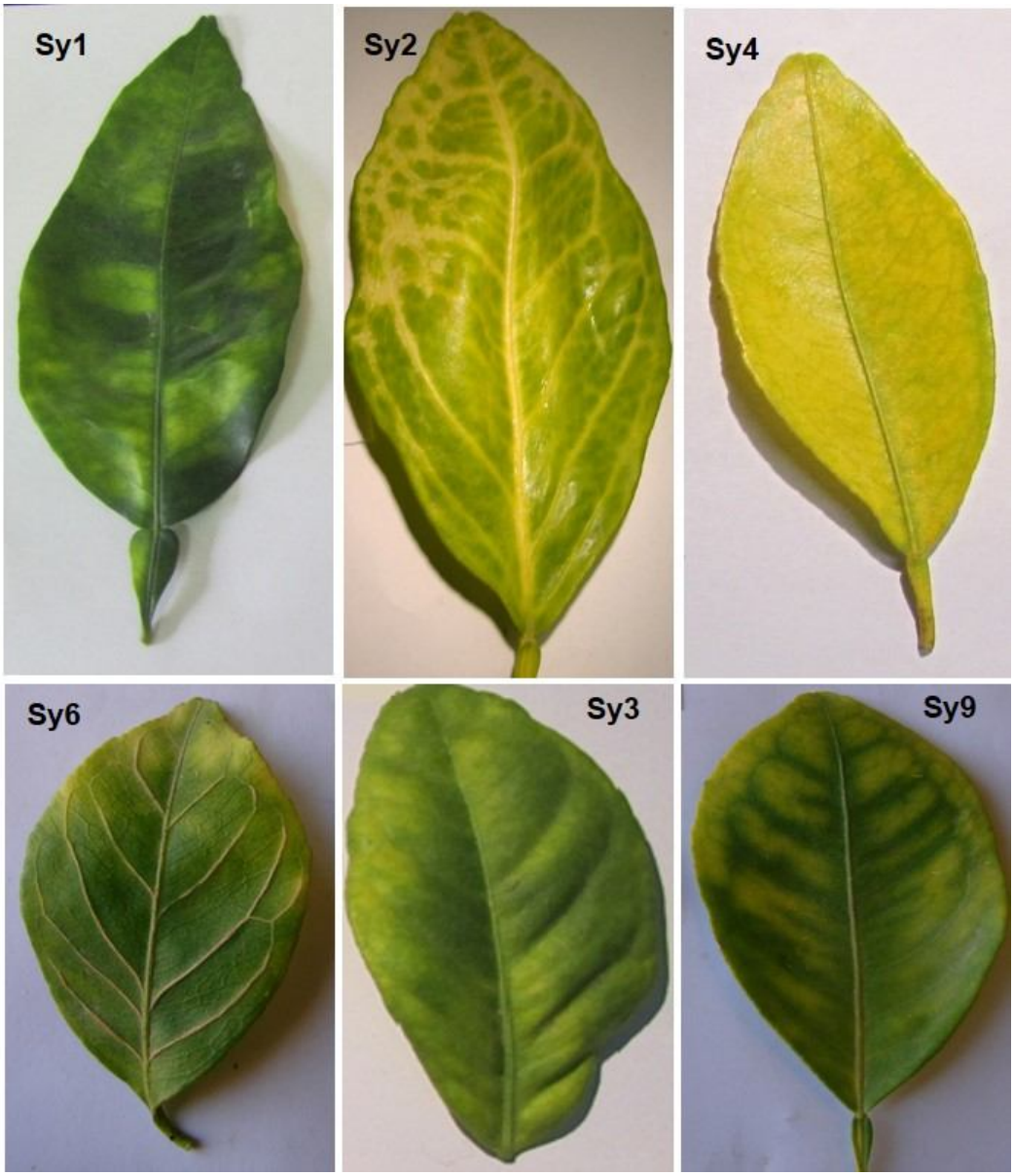

Figure 1 Foliar HLB-like symptoms in samples PCR positive for '*Ca.* Liberibacter asiaticus' (CLas +) and/or '*Ca.* Phytoplasma spp.' (Cphy +) Sy1: asymmetric blotchy leaf mottle (I2 strain; CLas +; Cphy-); Sy2: yellowing of the leaf veins (F3 strain; CLas +; Cphy-); Sy3 = yellow mottling, thickly, leathery leaves (S7 strain; CLas +; Cphy +); Sy4 = yellowing of the leaf and twig (Ba12 strain); Sy6: swollen, and corky leaf veins (N2 strain; CLas +; Cphy-); Sy9: yellowing between the veins (N6 strain; CLas-; Cphy +).

A partial rplA-rplJ gene sequence of CLas isolated from Sistan & Baluchestan (Sarbaz) demonstrated 100% sequence identity with two CLas isolates from Iran (accession nos. JF261098 and KP210470) and 99% nucleotide identity with some isolates from other countries including India and China. The ~1160bp amplcion of OI1/OI2C amplified in isolates from Sistan-Baluchistan (Pishin) (sample S4), Hormozgan (Siaho) (sample Ba6,) and Kerman (Jiroft) (sample N4) provinces resulted in high BLASTn matches with CLas sequences in

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GenBank database. Accordingly, the isolates of CLas detected in citrus samples were classified as strains of CLas.

Nested polymerase chain reaction (PCR) utilizing universal phytoplasma primer set P1/P7 followed by primer set fU5/rU3 identified 14 (18.18%) positive samples from symptomatic (HLB-like) samples but none from symptomless samples. Of these samples, eight were just positive for phytoplasma, including sweet orange (samples S3 and S6), lime (samples S5, S8, S9, and I21) and sweet lime (sample S10) from Sistan & Baluchestan, sweet orange (sample N6) from Kerman (Jiroft) and six for both phytoplasma and CLas, including lime (samples S7 and I20) from Sistan & Baluchestan and sweet orange (samples N1, N2, N4 and N5), from Kerman (Jiroft). No amplification was obtained in the negative control (symptomless) samples. These HLB-associated phytoplasma samples exhibited different symptoms such as yellow mottling, thickly and leathery leaves (samples S3, S5, S6, I20 and I21); yellowing of the leaf and twig (samples S8 and S9) and different patterns of blotchy mottle with swollen corky leaf veins (samples N1, N2, N4, N5) and/or yellowing between the veins (sample N6) (Fig. 1 and Table 3).

The detection rate of CLas was slightly higher in Kerman province (38.46%, 10/26 samples) compared to Sistan & Baluchestan province (33.33%, 11/33 samples).While, the detection rate of HLB-associated phytoplasma was lower in Kerman province (3.84, 1/26 samples) compared to Sistan & Baluchestan province (6.6%, 2/33 samples). No phytoplasma agent was detected in HLB symptomatic samples from Hormozgan province. The prevalence of CLas/phytoplasma co-infection in Kerman and Sistan & Baluchestan province were 15.38% (4/26 sweet orange samples, N1, N2, N4 and N5) and 6.6% (2/33 lime samples, S7 and I20), respectively.

In total, among 77 HLB symptomatic samples, 42.85 % of samples (33 samples) were CLas, 10.38 % (eight samples) were phytoplasma positive and six samples (7.79%) were positive for both HLB-associated phytoplasma and CLas indicating a mixed infection (Table 1). The rest of the samples exhibiting HLB symptoms, tested negative for CLas, *S. citri* and phytoplasma.

Molecular characterization of HLB associated phytoplasma

Nucleotide sequence analysis

Nucleotide sequences of six amplicons (samples N1, N2, N4, N6, N8 and I20) using primer sets fU5/rU3 and R16F2n/R16R2 were identical and resulted in high BLASTn matches with phytoplasma DNA sequences in GenBank database. The nucleotide sequences of the P1/P7 amplicons from strains N2 and N8 were identical to each other with a length of 1805 bp. The N2 strain sequence has been deposited in GenBank under accession no. KX348042 and the strain, was designated as HLBph-N2. The P1/P7 unique sequence of 1805 bp contained 1,521 bp of 16S rDNA at the 5′ end, and the entire 283 bp ribosomal intergenic region (RIR) plus part of 23S rDNA at the 3′ end. When the obtained sequence (1,805-bp) was used for BLAST search, the cactus witches'-broom (accession no. EU099561), Fars (Iran) alfalfa witches'-broom (acc. no. DQ233655), cotton phyllody phytoplasmas and *Ca.* Phytoplasma aurantifolia' (accession no. AF616637) all being members of group 16SrII showed the highest sequence identity of (99%).

Virtual RFLP analysis

Virtual RFLP analysis of 16S rDNA sequences was employed to assign HLB phytoplasma strains into subgroups (Wei *et al*., 2008). The results (Fig. 2) show that the the HLPy-N2 strain detected in Kerman (Jiroft) has a pattern identical to the reference pattern of the 16Sr group II '*Ca.* Phytoplasma aurantifolia' subgroup C (type strain of cactus witches' broom, accession no. AJ293216) (Fig. 2). The similarity coefficient of 0.99 indicates that HLPy-N2 is a variant of 16SrII-C subgroup (Table 4).

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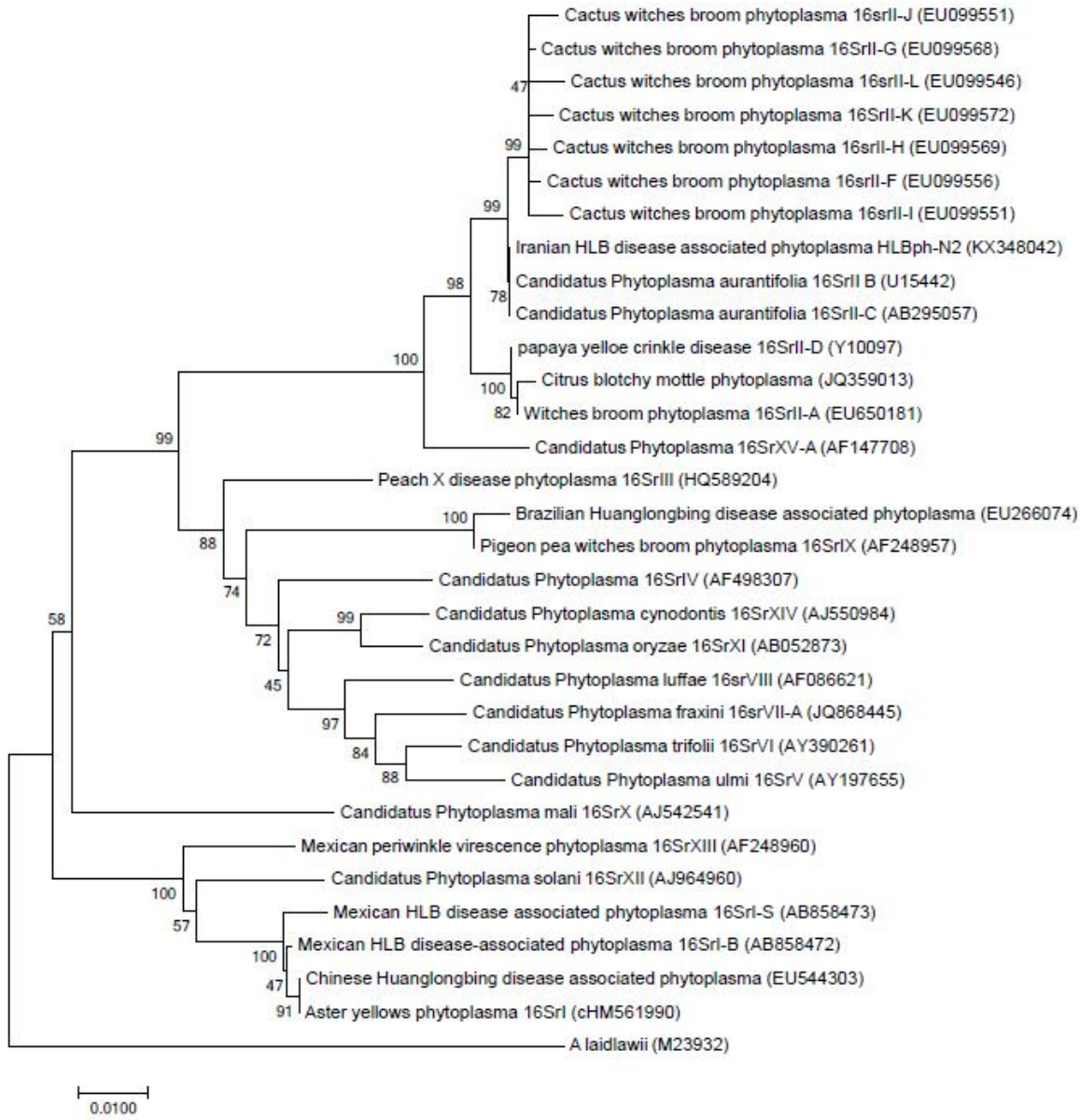

Figure 2 Phylogenetic tree constructed by neighbor-joining method based on the complete 16S rDNA sequences from the phytoplasma in this study (accession no. KX348042) and 30 other key phytoplasma groups and subgroups. Bootstrap values are shown on the branches. GenBank accession numbers for sequences are given in parentheses.

Phylogenetic analysis of complete 16S rDNA sequence

The complete 16S rDNA sequence of Iranian HLB associated phytoplasma (HLBph-N2 strain) was compared to 30 phytoplasma strains representing distinct phytoplasma 16Sr groups or subgroups, yielding the consensus tree shown in Fig. 3 This tree revealed that HLBph-N2 strain grouped with 16SrII group phytoplasmas. Among 16SrII group phytoplasmas,HLBph-N2 was closer to a strain of '*Ca*. Phytoplasma aurantifolia' (AB295057, 16SrII-C) than other 16SrII phytoplasmas including citrus_blotchy mottle phytoplasma

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(JQ359013) which was previously reported from China as a variant of 16SrII-A subgroup. Theother 15 phytoplasma strains were affiliated to other 16Sr groups. However, Brazilian HLB-symptom-associated phytoplasma (EU266074) clustered with a pigeon pea witches'-broom phytoplasma (AF248957) and Chinese HLB-symptom associated phytoplasma (EU544303) clustered with an aster yellow phytoplasma (HM561990).

Table 4 Similarity coefficients derived from analysis of virtual RFLP patterns of 16S rDNA R16F2n/R16R2 region from citrus HLP-phytoplasma strain and representative strains in 16SrII subgroups (A, B, C, D, E, F, G, H, I, J, K and L).

Serial	Strain/Accession no.	1	2	3	4	5	6	7	8	9	10	11	12
1	16SrII-A/L33765/	1.00											
2	II-B/U15442	0.88	1.00										
3	II-C/AJ293216	0.94	0.94	1.00									
4	II-D/Y10097	0.95	0.91	0.97	1.00								
5	II-F/EU099556	0.91	0.93	0.97	0.94	1.00							
6	II-G/EU099568	0.91	0.91	0.97	0.94	0.94	1.00						
7	II-H/EU099569	0.90	0.92	0.96	0.93	0.93	0.95	1.00					
8	II-I/EU099551	0.90	0.89	0.96	0.95	0.92	0.92	0.91	1.00				
9	II-J/EU099552	0.88	0.88	0.94	0.91	0.91	0.91	0.90	0.89	1.00			
10	II-K/EU099572	0.88	0.88	0.94	0.91	0.91	0.91	0.90	0.89	0.88	1.00		
11	II-L/EU099546	0.87	0.87	0.92	0.90	0.89	0.89	0.88	0.88	0.87	0.87	1.00	
12	HLB_like- phytoplasma	0.94	0.94	0.99	0.97	0.97	0.97	0.96	0.95	0.94	0.94	0.92	1.00

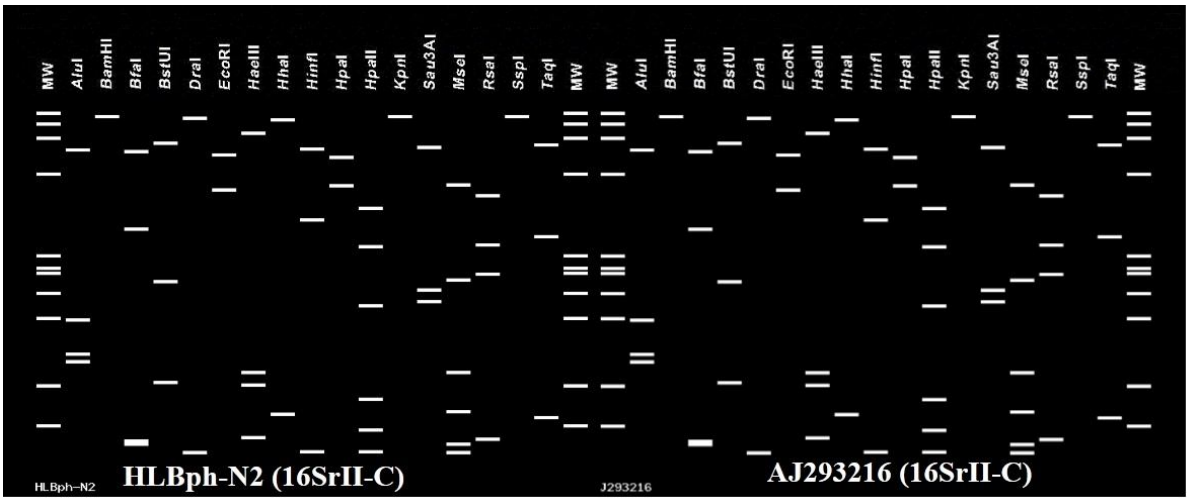

Figure 3 Distinct virtual RFLP patterns derived from in silico digestions of 16S rDNA 16F2n-R16R2 regions from the Iranian HLB-phytoplasma identified in this study (HLBph-N2) (accession no. KX348042) and 16Sr group II '*Ca.* Phytoplasma aurantifolia' subgroup C (type strain of cactus witches' broom, accession no. AJ293216). Recognition sites for the following 17 restriction enzymes were used in the simulated digestions: *Alu*I, *BamH*I, *Bfa*I, *BstU*I, *Dra*I, *EcoR*I, *HaeII*I, *Hha*I, *Hinf*I, *Hpa*I, *Hpa*II, *Kpn*I, *Sau3A*I, *Mse*I, *Rsa*I, *Ssp*I, and *Taq*I. MW = molecular weight standards from a φX174 DNA-*HaeIII* digestion.

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Discussion

Huanglongbing is a destructive disease of citrus that is now considered as an emergent and widespread threat to the Middle East and North Africa (MENA) citrus production (Wang and Trivedi, 2013). Until recently, the disease was only associated with the three species of *'Candidatus* Liberibacter' including CLas, '*Ca.* L. africanus' and '*Ca.* L. americanus' (Bové, 2006; Gottwald, 2010). However, other bacteria have recently been associated with HLB symptoms and are alternately identified as a phytoplasma strain belonging to '*Ca.* P. phoenicium' in Brazil (Teixeira *et al*., 2008), '*Ca*. P. asteris' and '*Ca.* P. aurantifolia' in China (Chen *et al*., 2009; Lou *et al*., 2014) and '*Ca* P. asteris' in Mexico (Arratia-Castro *et al.,* 2014).

HLB disease was observed throughout all three citrus growing areas in southern Iran: Sistan-Baluchistan, Kerman and Hormozgan provinces (Salehi *et al.*, 2012) and the presence of CLas in HLB-affected citrus trees has been confirmed employing DNA-based methods (Salehi *et al.*, 2012; Faghihi *et al.*, 2009).

In this study, both phytoplasma and CLas were detected in HLB-affected citrus as co-infection (7.79%) and single infection (10.38% for phytoplasma and 42.85% for CLas). Two CLas specific PCR primer pairs were used for single-step PCR and nested-PCR, and successfully detected the HLB pathogen in symptomatic citrus cultivars and hybrids including sweet orange, lime and tangerine. These results are in line with those of previous studies (Salehi *et al.*, 2012; Faghihi *et al.*, 2009, 2016; Golmohammadi *et al*., 2016). Furthermore, CLas was detected in HLB-like symptomatic citrus samples including sweet lime (sampleI3) and sour orange (samples I1, I2, SS1-SS4) from Sistan & Baluchestan (Sarbaz) (Table 3).

According to the proposed 16S rDNA-based phytoplasma classification scheme (Wei *et al.*, 2008), the HLB-associated phytoplasma from this study was considered as a member of peanut witches'broom (16SrII) phytoplasma group and therefore designated as a strain of '*Ca.* Phytoplasma aurantifolia'. By applying computer-simulated RFLP analysis and automated similarity coefficient calculation (Wei *et al*. 2007), similarity coefficients between representative strains of any two subgroups within a given 16Sr group should be equal to or lower than 0.97, and one or two asterisks (* or **) should be added after subgroup letters for denoting minor strains non-identical (giving a similarity coefficient F = 0.99 or 0.98) to the designated representative members in the corresponding subgroup. As the value of similarity coefficient between HLPy-N2 and cactus witches' broom phytoplasma (16SrII-C, accession no. AJ293216) reached 0.99 (Table 4), the HLPy-N2 from this study was considered as a variant (C*) of subgroup 16SrII-C. Nevertheless, the real RFLP analysis of 16S rDNA to determine the correct subgroup position of phytoplasma strains remains to be determined.

Based on analysis of 16S rDNA sequences, the Iranian HLB-phytoplasma (HLBph-N2) is different from the three previously reported HLB-symptom-associated phytoplasmas, 16SrIX in Brazil (Teixeira *et al.*, 2008), 16SrI in China (Chen *et al.*, 2009), and Mexican HLB disease-associated phytoplasma (AB858472), but it exhibited closer relation with citrus_blotchy mottle phytoplasma (JQ359013) strains that have been previously reported from China as a variant of phytoplasma subgroup 16SrII-A (Lou *et al.*, 2014).

In total, CLas detection rates were higher than those of HLB-associated phytoplasma. We determined both CLas and/or HLB-associated phytoplasma in 61% of symptomatic leaf samples, when compared to 48.9% as determined for different locations in China (Chen *et al.*, 2009) and 8.1% in Mexico (Arratia-Castro *et al.*, 2014). On the other hand, HLB-associated phytoplasma was detected in 10.4% of citrus plants showing HLB symptoms, and was less than the detected (78.0%) in a two-year study from different locations in China (Chen *et al.*, 2009) and a recent study in Mexico (23.3%) (Arratia-Castro *et al.*, 2014), but higher than that detected (8%) in China

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(Lou *et al.*, 2014). Th rate of detection both CLas and HLB-associated phytoplasma as co-infection was similar to another study (3.4-5.7%) (Arratia-Castro *et al.*, 2014).

Since some of the symptoms of citrus "Stubborn", a disease caused by the helical mollicute *S. citri* (Bové *et al.*, 2003), are somewhat similar to those of HLB, the samples were also tested with *S. citri*s specific primers. Stubborn is a disease of economic importance in all citrus growing regions of southern Iran (Khanchezar *et al.*, 2012) but no *S. citri* agent was detected in studied samples. Based on the PCR data, no positive test was found for CLas and phytoplasmas from 36 samples (46.7 %) exhibiting characteristic HLB symptoms, hence, the most common explanation is that the titer of CLas cells in the tested plant tissues is below the PCR detection limit. Another possibility is that disease symptoms are due to other causes (e. g., mineral deficiency). Nonetheless, it is also possible that other plant pathogens may also be involved in the etiology of HLB.

HLB-associated phytoplasma was detected in three citrus hosts, i. e., sweet orange (8 samples), lime (7 samples) and sweet lime (one sample). Until recently, the only identified phytoplasma of 16SrII group, associated with citrus disease, was '*Ca.* Phytoplasma aurantifolia', causative agent of witches'-broom disease, a strain of 16SrII-B subgroup of phytoplasma (Bové *et al.*, 2000). However, more recently, a phytoplasma of the subgroup 16SrIIA was characterized in a HLB-infected Grapefruit (*Citrus paradisi*) orchard, in Guangxi Province, China (Lou *et al*., 2014). Although host range of the '*Ca.* Phytoplasma aurantifolia' was earlier limited to acid Lime (*Citrus aurantiifolia*), it is now affecting several other citrus species and varieties like bakraee (*Citrus sp*.) (Djavaheri and Rahimian, 2004), Grapefruit (*C. paradisi*), citron (*C. medica*) (Azadvar *et al.*, 2015) and sweet orange (*C. sinensis*) (Azadvar, unpublished data) and mandarin (Salehi, personal communication).

The 1805 bp amplicon obtained with primer set P1/P7 of N8 phytoplasma strain showed 100% identity to N2 strain, although they were collected from different hosts, Bakraee and sweet orange and exhibited different symptoms, witches'-broom disease and HLB, respectively. Analyses of 16S rDNA sequences may not be sufficient to elucidate these strains relationships, therefore other molecular markers with higher resolving power such as the ribosomal protein operon (*rp*), the protein translocase subunit (*secY*), or the translation elongation factor (*tuf*) gene sequences (Contaldo *et al.*, 2015) are required. Bakraee is a local citrus variety in southern Iran (Golein *et al.*, 2012) and used as prevalent rootstock in southern citrus orchards in Iran. Witches'-broom of Bakraee was reported on bakraee in 2004 (Djavaheri and Rahimian, 2004). Accordingly, our results confirmed the association of phytoplasma of group 16SrII with other citrus hosts (sweet orange and sweet lime) showing HLB-like symptoms and phytoplasma association with sweet lime in Iran. Nevertheless, sweet limes exhibiting witches' Broom disease have also been reported in other regions eg. in Oman, UAE (El Shereiqi and Gassouma, 1993). Our results represent the first report of phytoplasma association with HLB in sweet lime in the world and first record of HLB and phytoplasma association with sour orange (*C. aurantium* L.) and sweet lime, respectively, in Iran. The insect vector of HLB-associated phytoplasma in Iran is not yet known but it could be a leafhopper, a planthopper, or even a psyllid.

HLB is one of the most serious threats to citrus worldwide and it seems the disease is spreading gradually into non infected areas in Iran (Salehi *et al.*, 2012) and threatening Turkey and beyond the Mediterranean Basin (da Graça *et al.*, 2016). Due to the destructive impact of the disease, more studies on other molecular and biological aspects of phytoplasma, as well as CLas agent are required. Such studies can enhance the design of efficient management strategies.

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بیماری باکتریایی مرتبط با هوانگ‌لونگ‌بینگ یا میوه سبز مرکبات در جنوب ایران

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چکیده: بیماری هوانگ‌لونگ‌بینگ (HLB) یا میوه سبز مرکبات یک بیماری مخرب مرکبات می‌باشد که امروزه به‌عنوان یک بیماری نوظهور و روبه گسترش در مناطق مرکبات‌کاری شمال افریقا و خاورمیانه محسوب می‌گردد. در بررسی که در سه استان مناطق مرکبات‌خیز جنوب کشور در طی سال‌های ۱۳۹۱-۱۳۹۲ انجام گرفت، عوامل باکتریایی *Candidatus* phytoplasma و *Candidatus* Liberibacter 'asiaticus'(CLas) به‌ترتیب در ۱۰/۳۸ و ۴۲/۸۵درصد و یا به‌صورت آلودگی توأم (۷/۷۹ درصد) در نمونه‌های جمع‌آوری شده با علایم *HLB* ردیابی شدند. براساس الگوی پیشنهادی برای رده‌بندی فایتوپلاسماها مبتنی بر 16S rDNA، عامل فایتوپلاسمایی مرتبط با نمونه‌های HLB در این مطالعه به‌عنوان یک واریانتی از زیر گروه 16SrII جاروک بادام‌زمینی لحاظ گردید این مطالعه اولین گزارش از ارتباط فایتوپلاسمایی با HLB در لیموشیرین در جهان و اولین گزارش از ارتباط CLas با لیموترش و نارنج در ایران می‌باشد.

واژگان کلیدی: *'Candidatus* Liberibacter asiaticus'، بیماری میوه سبز مرکبات، فایتوپلاسما، مرکبات، ایران

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